PHD

Actions of tumour necrosis factor-alpha in the rat isolated perfused heart

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Award date:
1998

Awarding institution:
University of Bath

Actions of Tumour Necrosis Factor-α in the Rat Isolated Perfused Heart

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for the degree of PhD

of the University of Bath

1998

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"The road goes ever on and on

Down from the path where it began.

Now far ahead the road has gone,

And I must follow, if I can,

Pursuing it with eager feet,

Until it joins some larger way

Where many paths and errands meet.

And whither then? I cannot say."

J. R. R. Tolkien

Summary

Tumour necrosis factor-α (TNF) may contribute to the depression in cardiac function often observed during septic shock, however potential mechanisms responsible for this remain unresolved. Increased circulating levels of TNF have also been associated with a variety of cardiac diseased states including chronic heart failure and myocardial ischaemia, and TNF may play a pathogenic role during these conditions. For these reasons the actions of TNF in the rat isolated perfused heart have been investigated. The major findings of this investigation are summarised below.

TNF was shown to attenuate insulin-stimulated glycogen synthesis, without altering insulin-stimulated glucose uptake. This effect could be mimicked by the cell permeable ceramide analogue, C_2-ceramide. Okadaic acid blocked the disruptions in insulin-stimulated glycogen synthesis seen with ceramide, however okadaic acid could not completely inhibit the actions of TNF on insulin-stimulated responses. TNF was also shown to have an early negative inotropic effect, which was accompanied by a coronary constriction. An attempt to characterise these responses with pharmacological probes was made. In this regard it was shown that the negative inotropic effect was probably the result of activation of the sphingomyelinase pathway. The coronary constriction was also appeared to be due to activation of the sphingomyelinase pathway, however, the phospholipase A_2 pathway, and in particular thromboxane A_2, were also involved in this action. Under certain conditions the direct negative inotropic effect and the coronary constrictor actions of TNF could synergise to cause a further depression in cardiac function.

Acknowledgements

Firstly I wish to thank Brian Woodward for his superb supervision and constant support over the past three years, but most of all I wish to thank him for his friendship.

I am very grateful to the British Heart Foundation who have funded this Ph.D. studentship, without whom none of this would have been possible.

I also want to thank my colleagues, past and present, within this laboratory, especially Kate, Su Fong, Darren and Harbans. All of these have been excellent people to work with and have made my time in Bath very enjoyable. I envy everybody who works with them in the future. I am also very grateful to everyone "downstairs" for their help in all things furry!

Thanks to my friends in Bath, especially Krish and Helen-back, for what else but friendship.

My parents deserve special recognition, they have been there for me forever, and especially over the last three years. Thank you for everything especially your understanding, encouragement and love.

And of course my dearest fiancée, Catherine, whose future I share and whose love and friendship has made everything worthwhile.

Contents

1 Introduction

1.1 Background

Tumour necrosis factor-α is a molecule which belongs to a large family of proteins termed cytokines. Cytokines are produced in response to a wide variety stimuli and are secreted by their producer cells, acting to alter the behaviour of target cells. Cytokines differ from the classical hormones, such as insulin, as they can be produced by a variety of different cell types and therefore tissues, where as hormones are produced by specific endocrine organs. Cytokines must normally bind to specific membrane receptors on the surfaces of target cells, and produce their effects by signal transduction across a plasma membrane. The actions of cytokines can be mediated in a number of different ways. Cytokines can act in an autocrine manner, whereby, if the producer cell expresses the required functional receptors, then the actions of the producer cell can be modified by the released cytokine. They can also act in a paracrine fashion, where the target cell is adjacent or close to the producer cell. Cytokines may also be released into the bloodstream and interact with distant target cells, thereby having an endocrine action.

1.1.1 Tumour necrosis factor

There are two types of tumour necrosis factor: tumour necrosis factor-α and tumour necrosis factor-β or lymphotoxin. Each share a number of related biological activities (Pennica *et al.*, 1984), bind to similar receptors and are approximately 30 % homologous to each other (Nedwin *et al.*, 1985). Tumour necrosis factor-β is lymphocyte-derived and is very important in various aspects of immune

responsiveness. However this project has focused on tumour necrosis factor-α (TNF), which is classically thought of as being macrophage-derived, although it is now recognised that TNF production is not exclusively synonymous with the macrophage and many other cells have been shown to produce TNF including cardiac myocytes (Kapadia *et al,*. 1995; Benigni *et al.*, 1996; Meldrum *et al,*. 1998; Wagner *et al.*, 1998). Many organs, including the heart, also have the potential to produce TNF due to the ubiquitous resident mast cell (Frangogiannis *et al.*, 1998)

1.1.2 Structure and release of TNF

TNF is secreted from producer cells as a protein with a molecular mass of 17 kDa (Beutler *et al.*, 1985a), consisting of 157 amino acids (in the human form). In macrophages, after TNF gene transcription, TNF mRNA is translated into a 26 kDa precursor to TNF in the cytoplasm. This 26 kDa precursor undergoes membrane insertation, where finally it is cleaved by TNF-α converting enzyme, releasing the mature 17 kDa TNF into the extracellular space (Moss *et al.*, 1997). Metalloproteinase inhibitors can suppress the TNF-α converting enzyme and inhibit TNF release (Mohler *et al.*, 1994). Biologically active TNF is composed of three 17 kDa TNF molecules in a noncovalently bound compact trimer (Jones *et al.*, 1989).

1.1.3 TNF receptors

Most, if not all, of the actions of TNF are secondary to TNF binding to specific cell surface receptors that are present on almost all cell types, with between 100 to 10,000 receptors per cell. Studies have identified two distinct TNF receptors, both of which

have been cloned and have molecular masses of 55 kDa and 75 kDa (Tartaglia & Goeddel, 1992), and have a lower and higher affinity for TNF, respectively. Crosslinking studies have demonstrated that the TNF trimer must bind with two or three receptor molecules to generate an intracellular signal (Tartaglia & Goeddel, 1992). The extracellular domains of the 55 kDa and the 75 kDa receptor share a 28 % sequence identity, however intracellular domains of each receptor show very little homology, suggesting activation of different signalling pathways. Polyclonal antibodies to both murine TNF receptors have been developed, and been shown to behave as receptor specific agonists. Studies with these antibodies have demonstrated that activation of each receptor signals two distinct TNF activities. The 55 kDa receptor is responsible for signalling cytotoxicity, and induction of several genes, whereas the 75 kDa receptor was shown to be responsible for the proliferation of primary thymocytes and a cytotoxic T-cell line (Tartaglia *et al.*, 1991). As mentioned earlier the 75 kDa receptor has a higher affinity for TNF than the 55 kDa receptor, and a ligand passing model between the two receptors has been suggested by Tartaglia *et al* (1993). In this model the 75 kDa receptor could augment the 55 kDa receptor mediated killing of murine L929 cells without itself generating a signal. The authors postulated that the 75 kDa receptor could increase the local concentration of TNF at the cell surface by rapid TNF association and disassociation, thereby producing an artificially high concentration of TNF at the 55 kDa receptor.

An interesting phenomenon concerning TNF receptor biology is the ability of these receptors to be shed in response to various stimuli, resulting in soluble TNF receptor type I (-55 kDa), and type II (-75 kDa) in the circulation of healthy humans, which are increased in a number of disease states, including sepsis (Girardin *et al.*, 1992; Van der

Poll *et al.*, 1993; Latini *et al.*, 1994). These shed receptors retain their ability to bind TNF and therefore can compete with cell surface TNF receptors for TNF binding (Kapadia *et al.*, 1995) and hence they can modulate TNF activity. The precise biological role for these shed receptors is uncertain, but it is thought to depend on the ratio of TNF soluble receptors and TNF protein, whereby low concentrations of soluble receptors could augment the biological effects of TNF by slowing degradation. Conversely, higher concentrations could act as TNF buffers, neutralising circulating levels of TNF (Tracey & Cerami., 1993).

1.1.4 TNF as a proinflammatory cytokine

TNF falls into a subset family of cytokines, known as proinflammatory cytokines, a group which also includes cytokines such as interleukin-1 (IL-1) and interferon-γ. Proinflammatory cytokines are key mediators in the hosts response to injury, inflammation and infection.

One of most important pro-inflammatory actions of TNF is activation of endothelial cells, leading to the active participation of these cells in an inflammatory response. TNF causes the induction and expression of various cell adhesion molecules on the surface of the endothelial cells, allowing binding of both neutrophils and mononuclear cells. Neutrophils can then be activated by TNF to increase superoxide generation (Laudanna *et al.*, 1993), thus increasing neutrophil mediated damage of cells. TNF can also stimulate endothelial cells to produce IL-1, prostacyclin, platelet activating factor and factors which exhibit pro-coagulant activities, all of which play important roles during inflammation (for review see Kunkel *et al.*, 1989). Activation of macrophages

by TNF inhibits intracellular replication of viral and parasitic organisms (Mestan *et al.*, 1986). Activated macrophages show increased cytotoxicity towards viral infected cells (Wong & Goeddal, 1986). Indeed, 55-kDa receptor knockout mice have been shown to have increased susceptibility to intracellular bacteria such as Listeria monocytogenes (Pfeffer *et al.*, 1993).

1.1.5 Signalling mechanisms of TNF

The multiple actions of TNF are mediated by a number of second messengers, and considerable effort has been devoted to clarifying the precise biological role for all of these. Available data are difficult to interpret since they have been obtained in a variety of different cells which respond in different ways to TNF. Many of the actions of TNF can be attributed to the degradation of membrane phospholipids, and subsequent generation of lipid second messengers. This section will describe the importance of the enzymes phospholipase C (PLC), phospholipase A_2 (PLA_2), and sphingomyelinase (SMase) in signal transduction by TNF.

1.1.5.1 TNF and phospholipase A_2 (PLA_2)

One of the most important signalling pathways utilised by TNF is the stimulation of phospholipase A_2 (PLA_2), which leads to the breakdown of membrane phospholipids to arachidonic acid (AA) and lysophospholipids. This action accounts for the proinflammatory actions of TNF. Once released by TNF, AA can be metabolised by cyclo-oxygenase enzymes to prostaglandins (Elias *et al.*, 1987) and thromboxane (Godfrey *et al.*, 1987), which are essential for the inflammatory process. In addition to

mediating the inflammatory responses to TNF, PLA_2 activation is thought to play an essential role in the cytotoxicity often seen with TNF (Suffys *et al.*, 1987; Hayakawa *et al.*, 1993).

An interesting study by Jayadev *et al.* (1994) linked the PLA_2 pathway to the metabolism of sphingomyelin and activation of SMase, where release of AA by PLA_2 could subsequently activate SMase. This is interesting as the SMase pathway is rapidly becoming recognised as another very important signalling mechanism utilised by TNF (see section 1.1.5.3).

1.1.5.2 TNF and phospholipase C (PLC)

Soon after the binding of TNF to the 55 kDa TNF receptor, TNF stimulates the production of diacylglycerol (DAG) from membrane phospholipids by the activation of PLC (Schutze *et al.*, 1992). Here generation of DAG was associated with a decline in phosphatidylcholine (PC) and increases in phosphorylcholine content, without changes in intracellular Ca^{2+}. These authors went on to show activation of a PC-specific PLC, where this PLC causes the production of DAG without accumulation of the Ca^{2+} mobilising inositol trisphosphate (IP_3) normally associated with PLC activation. Due to the lack of changes in intracellular calcium (Ca^{2+}) it is likely that the DAG produced activates a Ca^{2+}-insensitive isoform of protein kinase C (PKC). Also shown in this study was that the DAG produced could activate an acidic isoform of the SMase enzyme, and hence, initiate the SMase pathway (see section 1.1.5.3). Activation of PKC has also been observed by other groups, and is thought to mediate the actions of

TNF on the induction of JUN and FOS proteins, which are components of the AP-1 transcription factor (Brenner *et al.*, 1989).

1.1.5.3 The sphingomyelinase (SMase) pathway: relevance to TNF

As the SMase pathway is not as recognised as the pathways of PLA_2 and PLC, and because the SMase pathway is implicated in many of the actions of TNF described in this report, the following is an overview of the SMase signalling pathway. Comprehensive reviews of the degradation of sphingomyelin and subsequent second messenger functions of its metabolites have previously been published (Kolesnick, 1991; Merrill *et al.*, 1997).

Sphingomyelin is a phospholipid which is concentrated in the outer leaflet of the plasma membrane of most mammalian cells. The structure of sphingomyelin is shown in figure 1.1. When hydrolysed by SMase an immediate metabolite of sphingomyelin is ceramide. Ceramide can subsequently be converted, via the actions of ceramidase into sphingosine (structures of ceramide and sphingosine are also shown in figure 1.1). Both ceramide and sphingosine have potential functions as second messengers. Sphingosine can be converted to sphingosine 1-phosphate which also has biological activity. Interest in sphingolipids, of which sphingosine is one, arose when it was demonstrated that they could inhibit PKC (Hannun & Bell, 1987), and thus compounds which were initially thought to be inert were shown to alter the activity of a very important signalling molecule. An interesting observation at about the same time showed that DAG, the endogenous activator of PKC, could also activate SMase (Kolesnick, 1987). Thus it is possible that a negative feedback mechanism exists during

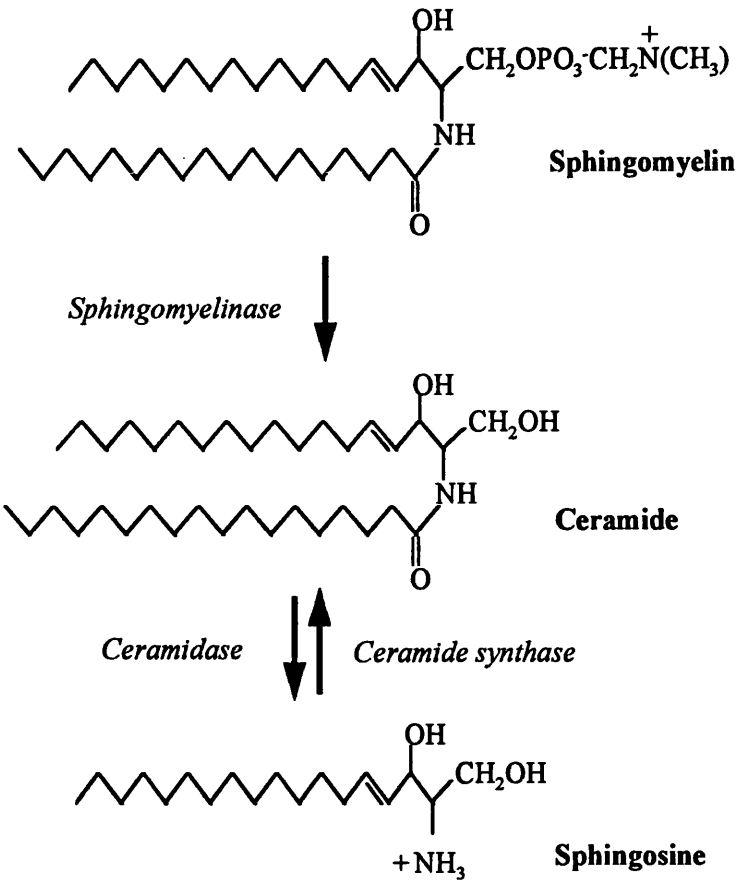


Figure 1.1. Molecular structures of sphingomyelin, ceramide and sphingosine. Also shown are the enzymes required for the breakdown of sphingomyelin to ceramide, and the breakdown of ceramide to sphingosine.

DAG stimulation of PKC, where DAG activation of SMase leads to the production of sphingosine, and inhibition of PKC. Therefore a negative feedback mechanism controlling overactivation of PKC by DAG may exist (Kolesnick, 1991). Many biological actions of ceramide, sphingosine and sphingosine 1-phosphate are now known, and some of these are discussed in later sections.

Identification of the SMase pathway as an important mediator of some of the biological actions of TNF came in 1991 (Kim *et al.*, 1991). It is now widely established that TNF can activate the SMase pathway. Indeed, TNF can activate the SMase pathway in a cell free system (Dressler *et al.*, 1992), suggesting tight coupling of the SMase enzymes to the TNF receptor, although as mentioned above prior release of AA may be required as an intermediary step in SMase activation (Jayadev *et al.*, 1994). The 55 kDa TNF receptor is thought to be responsible for activation of SMase (Weigmann *et al.*, 1992).

In a study by Candela *et al*, (1991) a situation was described where TNF-stimulated PLA_2 and cyclooxygenase enzymes were enhanced in the presence of low concentrations of sphingosine. This is especially interesting when taken in context of the study by Jayadev *et al.* (1994) and suggests that a self potentiating mechanism exists for TNF activation of the PLA_2 pathway through TNF-mediated generation of sphingosine. Also of interest was a recent study where exogenous sphingosine, or SMase caused a constriction in pig coronary artery which was blocked by the cyclooxygenase inhibitor, indomethacin (Murohara *et al.*, 1996). Thus, there appears to be intriguing interactions between the PLA_2 and SMase pathways.

1.2 TNF and insulin resistance

Increased plasma TNF levels, such as those which can occur during septic shock, cancer and various cardiac disease states (see below), have been associated with a general increase in the catabolic state of the body. A state of cachexia can develop, characterised by gross host wasting and a dramatic loss of body weight. Indeed, TNF was originally termed cachectin for its ability to induce cachexia. During cachexia decreased insulin sensitivity is common, a phenomenon which is also observed upon exogenous TNF administration (Lang *et al.*, 1992). In addition to this, TNF has been strongly implicated in the insulin resistance observed during obesity-induced diabetes and non-insulin dependant diabetes mellitus (NIDDM). In obesity-linked insulin resistance models or NIDDM models adipocytes appear to overproduce TNF, which if neutralised can increase peripheral uptake of glucose in response to insulin (Hotamisligil *et al.*, 1993). Also TNF deficient mice are protected from obesity induced insulin resistance (Uysal *et al.*, 1997).

The heart is an organ which requires a great deal of energy to fulfil its job of constantly pumping blood around the body. Although the preferred "fuels" for the heart under physiological conditions are circulating free fatty acids (normally providing 60 - 70 % of energy needs), the heart still has a large capacity to utilise glucose as a substrate. In this regard the heart is an organ which is very sensitive to insulin. Two of the major actions of insulin, on carbohydrate metabolism, in hearts are to increase glucose uptake by cardiac myocytes and to increase glycogen synthesis within these cells. Glycogen is an important endogenous energy store for the heart, which acts as a reserve of energy

to support sudden increases in heart work and may also contribute to survival and recovery of hearts after a periods of substrate deprivation, such as ischaemia (Cross *et al.*, 1996). Thus, any disruption in the actions of insulin could have important consequences for the heart.

In order to appreciate the potential mechanisms behind TNF-induced insulin resistance it is first necessary to understand glucose uptake and metabolism within cells. It is also important to understand the signal transduction pathways utilised by insulin.

1.2.1 Glucose uptake and metabolism

It has long been known that the plasma membranes of virtually all mammalian cells possess a transport system for glucose (GLUT transporters), which allow the movement of glucose down its concentration gradient either into or out of cells. The glucose concentration in the extracellular space is so much greater than in the cytosol that facilitated diffusion under physiological conditions is always into the cell. GLUT transporters are stereospecific for the D-enantiomer of glucose and require no additional energy input, such as ATP, for their actions (Baldwin & Lienhard, 1981). It has long been established that perfused rat hearts are sensitive to insulin, increasing glucose uptake upon insulin administration (Neely & Morgan, 1974). Although there is a large family of glucose transporters, only two are expressed in cardiac tissue, GLUT1 and GLUT4. GLUT transporter proteins exist within cells in tubulo-vesicular structures and undergo a cycle of exocytosis to the plasma membrane, and endocytosis from the plasma membrane, back to their intracellular pool (figure 1.2; Gould &

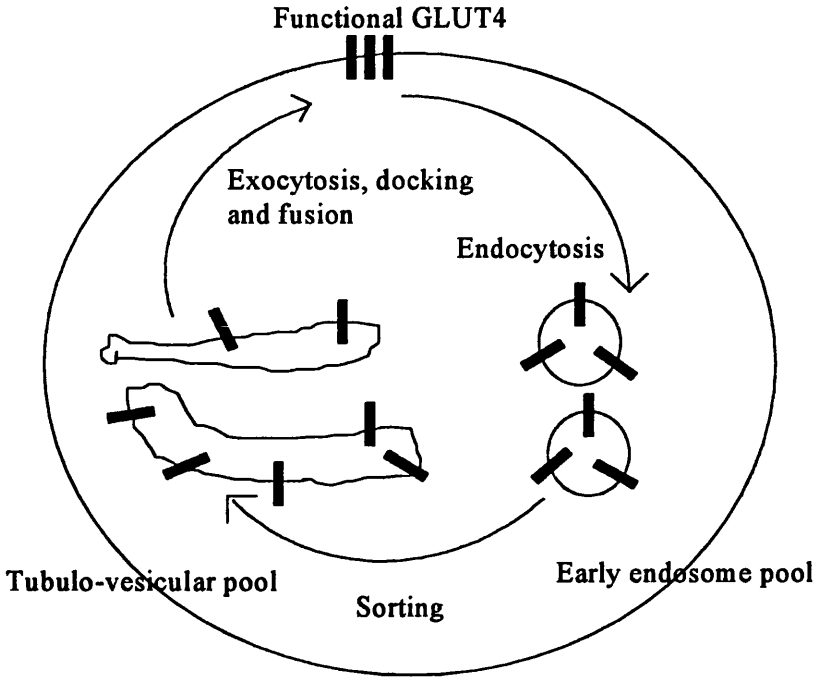


Figure 1.2. The cycling of the GLUT4 glucose transporter within insulin sensitive cells.

Holman, 1993). This cycle is thought to be constantly active, and changes in the cell surface GLUT concentration occur by increasing or decreasing endocytosis or exocytosis. GLUT1 is the transporter responsible for basal glucose uptake, and is constitutively expressed at high levels at the plasma membrane. GLUT1 is only weakly sensitive to insulin (Yang & Holman, 1993) and it is generally regarded as insulin-independent. GLUT4 has very low basal plasma membrane expression and resides mainly within the tubulo-vesicular pool. Insulin stimulation leads to an increase in the exocytosis of GLUT4 to the plasma membrane and, therefore, increased glucose uptake (figure 1.2; Satoh *et al.*, 1993). Therefore, GLUT4 is classically recognised as the insulin-sensitive glucose transporter.

Once glucose enters the cell, it is rapidly phosphorylated to glucose 6-phosphate (G-6-P) via the action of hexokinase. Phosphorylation of glucose serves three major purposes; firstly, phosphorylation turns the glucose molecule into a negatively charged ion, which cannot leave the cell; secondly, it serves to "prime" the glucose molecule for metabolism via glycolysis; and thirdly, phosphorylation of glucose maintains the concentration gradient for glucose entry into the cell. G-6-P can then enter into the glycolytic pathway or it can be converted to the endogenous glucose store, glycogen. During glycolysis, glucose is transformed by a host of reactions to two molecules of pyruvate, producing a net of two molecules of ATP and two molecules of NADH. The fate of pyruvate is then decided by the oxygen (O_2) levels within the heart. If O_2 is sufficient then pyruvate enters into the citric acid cycle, where the energy stored within the molecule is fully realised. If the heart is operating under anaerobic conditions then

pyruvate is converted to lactate via the lactate dehydrogenase reaction, and lost through the cell wall (Katz, 1992a).

As mentioned above G-6-P can also be converted to glycogen. In this process, G-6-P is first converted to glucose 1-phosphate (G-1-P) by the enzyme phosphoglucomutase. G-1-P is the initial substrate required for glycogen synthesis as well as being the product of glycogen breakdown. Glycogen synthesis and breakdown are separate processes which are both tightly controlled and mediated by two highly regulated enzymes. Glycogen synthase (GS) is responsible for glycogen formation and phosphorylase mediates glycogen breakdown. Both of these enzymes exist in active and inactive forms. The initial reaction in the synthesis of glycogen involves the conversion of G-1-P to UDP-glucose, utilising the high energy phosphate bond of UTP, this reaction is not rate limiting. The glucose moiety is then transferred to glycogen via the action of GS, a reaction which is rate limiting, and depends on the activation state of the GS enzyme. GS exists in two activation states, which are controlled by phosphorylation reactions, the dephosphorylated form being more active that the phosphorylated one (Roach *et al.*, 1976). Phosphorylation of GS by PKA is thought to be one of the mechanisms by which β-adrenergic agonists stimulate glycogen breakdown (Dent *et al.*, 1990). G-6-P levels are also very important to glycogen synthesis, as G-6-P is an endogenous allosteric activator of GS, and with a high concentration of G-6-P nearly full GS activity can be observed even when GS is in its less active state (Roach & Larner, 1976).

Phosphorylase, the enzyme responsible for the degradation of glycogen, is also controlled by its phosphorylation state, but in this case the phosphorylated form is the

more active one (Madsen, 1986). Again PKA can phosphorylate this enzyme, causing activation and hence stimulate glycogen breakdown. Activity of phosphorylase is controlled by yet another enzyme, phosphorylase kinase. Phosphorylase kinase itself is activated by phosphorylation and deactivated by dephosphorylation (Pickett-Gies & Walsh, 1986). The process of glycogen synthesis and breakdown is shown in figure 1.3.

The glycogen associated form of protein phosphatase-1 ($PP\text{-}1_G$) is an enzyme which is very important in the regulation of both GS and phosphorylase kinase activities, when activated by phosphorylation, it causes the dephosphorylation of both GS (activating it), and phosphorylase kinase (deactivating it), with the effect of stimulating glycogen synthesis and inhibiting glycogen breakdown respectively (figure 1.3; Dent *et al.*, 1990). Interestingly $PP\text{-}1_G$ has been shown to be phosphorylated and therefore activated by $p90^{rsk}$ (MAPKAP kinase I), thereby linking glycogen metabolism to the MAP kinase pathways (Sutherland *et al.*, 1993). Glycogen synthase kinase-3 (GSK3), is another important regulatory enzyme, which causes the phosphorylation and therefore inactivation of GS (Hughes *et al.*, 1993). Moreover, it appears that GSK-3 is constituatively active, resulting in basal phosphorylation and therefore basal inhibition of GS (Hughes *et al.*, 1993). Most of these studies have been conducted in either adipocytes or in skeletal muscle, therefore, interpretation of these results need to be extrapolated into the heart, however almost certainly differences exist between the heart and these other tissues.

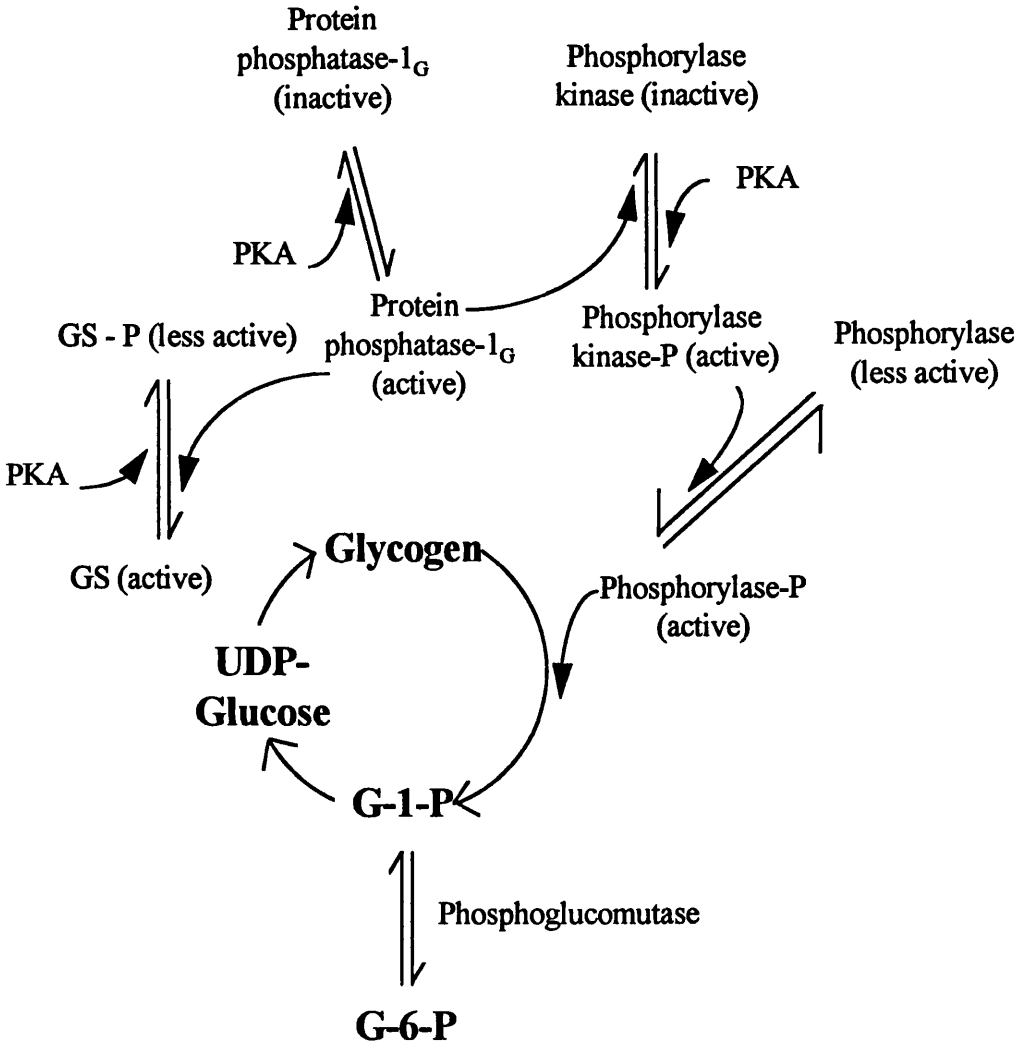


Figure 1.3. A diagrammatical representation of the enzymes controlling glycogen synthesis and breakdown. Abbreviations: Glycogen synthase, GS; protein kinase A, PKA; glucose 6-phosphate, G-6-P; glucose 1-phosphate. G-1-P.

1.2.2 Insulin signal transduction and effects on glucose metabolism

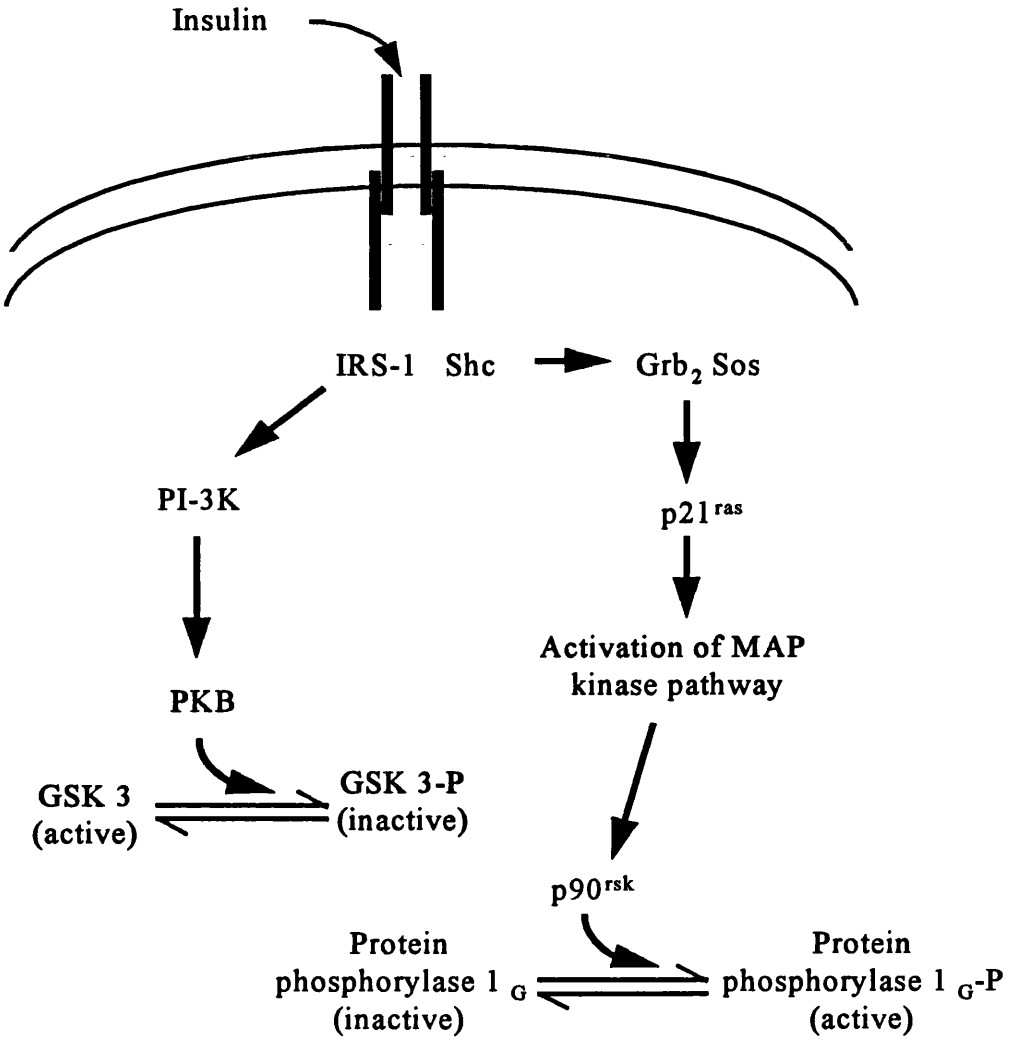
Binding of insulin to its receptor causes the receptor to undergo autophosphorylation which significantly increases catalytic activity of the receptor leading to tyrosine phosphorylation of cellular substrates (Wilden *et al.*, 1992). In this manner, insulin stimulates a variety of different signal transduction pathways leading to protein synthesis, gene expression, cell growth and changes in glucose transport and metabolism. However, this section will concentrate on the pathways thought to be involved in glucose transport and metabolism.

Insulin receptor substrate-1 (IRS-1) is probably the most important, and certainly most extensively studied substrate of the insulin receptor, although IRS-2 and IRS-3 also exist (Tsuji *et al.*, 1998). IRS-1 has been widely implicated in the actions of insulin on glucose transport (Kanai *et al.*, 1993; Holman & Cushman, 1994; White & Kahn, 1994). When phosphorylated, IRS-1 can bind other cytoplasmic signalling molecules that contain Src homology 2 (SH2) domains. This enables IRS-1 to associate with the p85 regulatory subunit of phosphatidylinositol 3'-kinase (PI-3K), activation of PI-3K thus ensues (Myers *et al.*, 1992). The specific PI-3K inhibitor, wortmannin, has been shown to inhibit insulin stimulated glucose uptake (Kanai *et al.*, 1993). IRS-1 may act to localise PI-3K to low density membrane fractions, which is thought to represent the GLUT4 containing tubulo-vesicular pool (Yang, *et al.*, 1996). The formation of phosphatadylinositol 3,4,5-trisphosphate, which is a product of PI-3K activation, could alter the nature of the lipids in the intracellular vesicles or the plasma membrane increasing the probability of fusion and docking.

Another major action of insulin, and one which has not been very well characterised, is its ability to stimulate the synthesis of glycogen. As mentioned earlier, two very important enzymes in glycogen metabolism are PP-1_G and GSK-3. Insulin acts to inhibit GSK-3 and to stimulate PP-1_G, thus causing glycogen synthesis (Dent *et al.*, 1990; Sutherland *et al.*, 1993). Although there is much controversy concerning the precise mechanism responsible for insulin stimulated glycogen synthesis, two main pathways have been outlined, these are summarised in figure 1.4.

In 1990, Dent *et al*, proposed a theory that upon insulin stimulation, a protein kinase was activated, which they termed insulin stimulated protein kinase-1 (ISPK-1). ISPK-1 was shown to have the ability to cause phosphorylation of PP-1_G, thereby inactivating phosphorylase kinase and therefore decreasing glycogen breakdown. As expected, PP-1_G activation also caused activation of GS, stimulating glycogen synthesis. ISPK-1 was subsequently shown to be $p90^{rsk}$ (Sutherland *et al.*, 1993; figure 1.4).

Thus, the first model for insulin-stimulated glycogen synthesis was proposed to account for these effects on glycogen synthesis, whereby insulin causes tyrosine phosphorylation of Shc protooncogene which induces its association with growth factor receptor-bound protein 2 (Grb2). Grb2 can then target the nucleotide exchange factor, Son of sevenless (Sos), leading to the binding of GTP to $p21^{ras}$ and, thus, stimulation of the mitogen activated protein kinase (MAP kinase) cascade, and subsequent stimulation of $p90^{rsk}$ (this potential pathway is outlined in a recent review by Lawrence & Roach, 1997). This pathway, however, has been disputed. Weise *et al.*,



(1995) showed that similar activation of the MAP kinase cascade by epidermal growth factor, was not associated with any major changes in glycogen synthesis. Also the

Figure 1.4. Potential mechanisms of insulin-induced glycogen synthesis. Abbreviations: Insulin receptor substrate-1, IRS-1; phosphatidyl inositol 3-kinase, PI-3K; protein kinase B, PKB; glycogen synthase kinase 3, GSK3; growth factor receptor-bound protein 2, Grb_2; son of sevenless, Sos.

MAP kinase inhibitor PD 098059, could not block insulin stimulated activation of GS (Azpiazu *et al.*, 1996).

The other potential mechanism for insulin-induced glycogen synthesis, like insulin-induced glucose uptake, involves activation of IRS-1 and PI-3K. Here the target molecule for the insulin signal is GSK-3. In this proposed model insulin inhibits the high basal activity of GSK-3 causing activation of GS and, therefore, increased glycogen synthesis. Phosphorylation and inhibition of GSK-3 by insulin is mediated by a relatively newly discovered kinase, protein kinase B (PKB), which is activated by PI-3K (figure 1.4; Cross *et al.*, 1995; Cross *et al.*, 1997). This pathway is also reviewed by Lawrence & Roach (1997).

1.2.3 Potential mechanisms responsible for TNF mediated insulin resistance

Since the realisation that TNF is an essential mediator of the insulin-resistance observed during obesity-induced diabetes (Hotamisligil *et al.*, 1993), and therefore could play an important role in NIDDM, research into the mechanisms behind TNF-induced insulin resistance has almost grown into a field of its own. Studies have taken place almost exclusively in cultured or isolated cell lines. Several, often contradictory, mechanisms have been suggested and these will be reviewed in the following section.

Prior to the suggestion that TNF may mediate obesity-induced diabetes a study by Stephens & Pekala (1991) showed that in 3T3-L1 adipocytes chronic (20 hr) treatment lead to an 85 - 90 % decrease in mRNA for the insulin-sensitive GLUT4 transporter, and the authors postulated that the actions of TNF were at least in part at the level of transcription. This observation was subsequently confirmed in the initial study implicating TNF in obesity-induced diabetes (Hotamisligil *et al.*, 1993), where 10 days treatment of adipocytes with TNF resulted in a marked decrease in the expression of GLUT4. Increased TNF mRNA was observed in adipose tissue from obese, insulin resistant animals (Hotamisligil *et al.*, 1993). In addition to this, administration of anti-TNF antibodies increased glucose uptake.

The first evidence for TNF induced alterations in insulin signalling was also presented in 1993 (Feinstein *et al.*, 1993), where in hepatoma Fao cells 60 min exposure to TNF suppressed insulin-induced tyrosine autophosphorylation of the insulin receptor, this was accompanied by decreased tyrosine phosphorylation of IRS-1. These changes were not due to alterations in insulin binding to its receptor. Evidence implicating changes in the tyrosine kinase activity of the insulin receptor, and so furthering the observations by Feinstein *et al*, came a year after (Hotamisligil *et al.*, 1994a; Hotamisligil *et al.*, 1994b). In the first of these studies, decreased tyrosine kinase activity of the insulin receptor was seen during obesity-induced diabetes in both muscle and fat cells, and was accompanied by decreased phosphorylation of IRS-1. Neutralising antibodies to TNF caused a marked increase in insulin-stimulated autophosphorylation, and subsequent phosphroylation of the IRS-1 (Hotamisligil *et al.*, 1994a). The second of these studies showed that in adipocytes, 5 days treatment with

TNF caused a decrease in insulin-stimulated glucose uptake, which was accompanied again by decreased insulin receptor autophosphorylation and decreased phosphorylation of IRS-1 (Hotamisligil *et al.*, 1994b).

Kanety *et al* (1995), showed that only 60 min treatment of Fao cells with TNF resulted in a decrease in insulin-stimulated tyrosine phosphorylation of IRS-1, however this was not associated with a reduction in insulin-receptor tyrosine kinase activity. Decreased phosphorylation of IRS-1 resulted in impaired association of IRS-1 with PI-3K. Interestingly, TNF caused increased serine phosphorylation of IRS-1 which lead to a decrease in the electrophoretic mobility of IRS-1, and could have further impaired association with PI-3K. This increase in serine phosphorylation was mimicked by inclusion of okadaic acid or calyculin A. As these compounds have been shown to inhibit phosphatase enzymes (Bialojan, & Takai, 1988; Ishihara *et al.*, 1989), it was suggested that the actions of TNF on IRS-1 were due to inhibition of a serine/threonine phosphatase. Thus, increased serine phosphorylation of IRS-1 impaired its activation by the insulin receptor (Kanety *et al.*, 1995). Serine phosphorylation of IRS-1 induced by TNF was observed again in 1996 (Hotamisligil *et al.*, 1996). In cultured adipocytes the serine phosphorylated form of IRS-1 was shown to actually act as an inhibitor of the insulin receptor tyrosine kinase activity. A similar inhibitory form of IRS-1 was also observed in fat and muscle tissue from obese, insulin resistant animals (Hotamisligil *et al.*, 1996), and a soluble TNF receptor fusion protein could ameliorate this.

Another study, this time using antibody agonists specific for each TNF receptor, implicated the 55 kDa TNF receptor in TNF mediated insulin resistance (Peraldi *et al.*,

1996). An important finding in this investigation was that serine phosphorylation of IRS-1 could be mimicked by exogenous addition of either sphingomyelinase (SMase) or the cell permeable analogues of ceramide, C_2-ceramide and C_6-ceramide (Peraldi *et al.*, 1996). Again this resulted in an inhibitory form of the IRS-1 molecule with respect to the insulin receptor. The 55 kDa receptor for TNF is known to be coupled to the SMase pathway (section 1.1.5.3), implying that the actions of TNF on IRS-1 are mediated by ceramide. Indeed, ceramide has been shown to activate a kinase termed ceramide-activated protein kinase (CAPK), capable of serine/threonine phosphorylation (Mathias *et al.*, 1991).

Evidence for implicating the SMase pathway in TNF-induced insulin resistance has been presented by another group, however in this study, a completely different mechanism of action was suggested (Begum & Ragolia, 1996). Here, 60 min exposure of a cultured skeletal muscle cell line to TNF resulted in a decrease in insulin-stimulated glucose uptake as well as a decline in glycogen synthase activity. TNF blocked insulin activation of PP-1_G, and it appeared that the action of TNF on glycogen synthesis was more potent than its effect on insulin-stimulated glucose uptake. Again this could be mimicked by SMase and cell permeable ceramide analogues. Ceramide is capable of activating a phosphatase termed ceramide-activated protein phosphatase (CAPP), a member of the PP-2A family of phosphatase and, therefore, potently inhibited by okadaic acid (Dobrowsky & Hannun, 1992), PP-2A can dephosphorylate PP-1_G, therefore it could interfere with insulin stimulation of PP-1_G (Dent *et al.*, 1990). PP-2A can also dephosphorylate GSK3, thereby reversing insulin induced GSK3 inactivation (Murai *et al.*, 1996), both of these actions would inhibit insulin-stimulated glycogen synthesis.

Yet another suggested mechanism of TNF-induced insulin resistance involves the activation of a tyrosine phosphatase, which would directly deactivate the insulin receptor and inhibit IRS-1 activation (Kroder *et al.*, 1996). Conversely, it has been shown that 15 min exposure of 3T3-L1 adipocytes to TNF lead to increased tyrosine phosphorylation of IRS-1, and thus increased the association of IRS-1 to PI-3K in response to insulin (Guo & Donner, 1996), suggesting a pro-insulin effect of TNF. However, insulin-stimulated glucose uptake was not measured in this study. Chronic treatment with TNF for 5 days, did lead to a disruption in the insulin receptor signal. Also, another study failed to observe any effect of TNF on insulin-stimulated glucose uptake or glycogen synthesis in isolated rat soleus muscle strips regardless of exposure time (Furnsinn *et al.*, 1997).

Therefore, it is clear that although there has been extensive work investigating the interactions between TNF and insulin the story is still quite confused. Current theories suggest inhibitory actions of TNF on the insulin receptor, its direct substrates (IRS-1) or even at more distal targets of the insulin signal. Specific actions of TNF are probably dependant on the tissue or cell studied, however to my knowledge there has been no investigations concerning the actions of TNF on insulin-stimulated responses in cardiac tissue.

1.3 Septic shock

Septic shock is a state which is characterised by fever, profound hypotension, decreased cardiac function and increased systemic vascular resistance, resulting in renal, hepatic and cerebral injury, finally death can ensue due to multi-organ systems failure or respiratory arrest. Many of the deleterious consequences of septic shock can be mimicked by injecting endotoxin, which is a lipopolysaccharide (LPS) component of the cell wall in certain bacteria.

1.3.1 Septic shock, and the role of TNF

It is widely accepted that TNF plays a central role in the pathophysiology of septic shock, and its experimental counterpart, endotoxin shock. Indeed, it has long been known that endotoxin is one of the most potent inducers of TNF release from macrophages (Beutler *et al.*, 1985a), and it is now apparent that other cell types, including cardiac myocytes and resident cardiac mast cells, are capable of producing significant amounts of TNF in response to endotoxin (Giroir *et al.*, 1992; Kapadia *et al.*, 1995; Saghizadeh *et al.*, 1996; Frangogiannis *et al.*, 1998).

Early release of TNF into the circulation in response to a Gram-negative bacterial lipopolysaccharide challenge was first described by Beutler *et al* in 1985 (Beutler *et al.*, 1985b). Subsequent studies have shown that immunisation of animals against TNF can produce marked protection from many of the pathophysiological consequences of an endotoxic challenge or direct *E. coli* administration (Beutler *et al.*, 1985c; Tracey *et*

al., 1987; Mathison *et al.*, 1988). These protective effects resulted in increased survival of experimental animals. The study by Tracey *et al.* (1987) showed that anti-TNF antibodies protected against some of the profound cardiovascular consequences of endotoxin administration, such as decreased cardiac output and severe hypotension. In 1986, Tracey *et al.* showed that infusion of recombinant TNF into mammals could mimic the effects of endotoxin, inducing hypotension and shock. Histological examination after death showed ischaemic lesions of the gastrointestinal tract, and acute renal tubular necrosis, suggesting increased systemic vascular resistance and inadequate vital organ perfusion. In addition to this, administration of antibodies to TNF attenuates the appearance of other cytokines involved in septic shock syndrome, i.e. interleukin-1 and interleukin-6 (Fong *et al.*, 1989).

Thus, three major lines of evidence suggest that TNF is a causal factor in septic shock syndrome: firstly, it is one of the earliest mediators secreted by the host in response to bacterial or endotoxic challenge; secondly, administration of recombinant TNF causes a shock-like state; and finally, neutralisation of TNF with anti-TNF antibodies can attenuate the release of other mediators of septic shock, and effect marked protection from a lethal endotoxic challenge.

1.3.2 Depressed cardiac function during septic shock

As mentioned earlier, one of the major characteristics of septic shock is profound hypotension with depressed cardiac function. This depressed cardiac function could potentially contribute to the hypotension observed, and hence to under-perfusion of vital organs, and eventually multiple-organ systems failure.

Abnormal cardiac function during shocked states has been observed on numerous occasions, and has been recognised for many years (Solis & Downing, 1966; Starzecki & Spink, 1968). Although it is generally appreciated that there is depressed cardiac function, the precise time course for this is not so certain. Some investigators have observed myocardial depression within 30 - 60 min (Solis & Downing, 1966; Guntheroth *et al.*, 1982), while other investigators have described late (> 4 hr) myocardial dysfunction (Hinshaw *et al.*, 1974; Starzecki & Spink, 1968). Depressed cardiac function in patients is thought to be reversible, whereby patients surviving septic shock show a return to normal cardiac contractility by about 10 days after the onset of shock (Parker *et al.*, 1984).

Various studies have been performed with the aim of characterising changes in cardiac function after endotoxin administration. It does not appear likely that endotoxin itself can abnormally alter cardiac contractility, as addition of 1000 $\mu g.ml^{-1}$ did not alter tension developed by a guinea-pig atrial muscle preparation after 90 min incubation (Parker & Adams, 1979). However, depression was seen when hearts were removed from animals treated with endotoxin, suggesting the release of a myocardial depressant substance (Parker & Adams, 1979). Further evidence for a circulating myocardial depressant substance during shock came from studies by Parrillo *et al.* (1985), where sera from septic patients could depress *in vitro* cardiac myocyte performance. The depression in cardiac myocyte performance was obvious within minutes of perfusion with sera from septic patients, but not evident at all during perfusion with sera from healthy volunteers or post-shock patients. Cardiac myocytes, isolated 4 hr after *E. coli* LPS administration to guinea pigs, were shown to have depressed contractile activity,

which was not due to altered myofilament Ca^{2+} responsiveness, but probably a consequence of a decrease in the Ca^{2+}-transient during systole (Rigby *et al.*, 1998). Earlier the same group had demonstrated reduced L-type calcium currents in ventricular cardiac myocytes isolated 4 hrs after LPS administration to guinea pigs (Zhong *et al.*, 1997). Herbertson *et al.* (1996) showed that decreased left ventricular contractility 4 hr after endotoxin could be partially attenuated by nitric oxide (NO) synthase inhibition in the pig. Although a role for NO in the depressed contractility seen in isolated perfused hearts *ex vivo* after LPS treatment was not found (Decking *et al.*, 1995).

One of the potential mechanisms for depressed myocardial performance during sepsis involves decreased sensitivity, or surface expression, of β-adrenergic receptors (Eisinger *et al.*, 1988; Yasuda & Lew, 1997). Although it is unlikely that this mechanism would be important during the *in vitro* depressed cardiac myocyte performance with sera from septic patients (Parrillo *et al.*, 1985) or the decreased inotropy of *ex vivo* heart muscle preparations (Parker & Adams, 1979) mentioned earlier, in whole animals it could be important. Decreased β-adrenoceptor responsiveness, whether through decreased sensitivity or β-adrenergic receptor down-regulation, in the whole animal would lead to altered inotropic responses to circulating catecholamines, and, hence, would be expected to be observed as a depression in cardiac function.

An interesting study by Hohlfeld *et al.* (1995) showed that 3 hr after LPS administration to rats, *ex vivo* perfusion of hearts from these animals lead a marked increase in coronary tone when compared to control hearts. The authors showed that

this coronary vasoconstriction was due to endothelin-1 (ET-1). Although ET-1 would be unlikely to have a direct negative inotropic action (Kramer *et al.*, 1991), coronary constriction would decrease substrate supply to the heart, and, hence, compromise cardiac function in an indirect manner. In this study it was also shown that this increased coronary resistance could be blocked by anti-TNF antibodies when administered 30 min prior to LPS.

1.3.3 TNF as a myocardial depressant in septic shock

Tracey *et al.* (1986) showed that many of the cardiovascular alterations observed during septic shock could be mimicked by TNF. In a later study Tracey *et al.* (1987) showed that antibodies against TNF could protect against the decreased cardiac output seen after endotoxin. In addition, TNF has been shown to cause a decrease in cardiac function *in vivo* in dogs (Pagani *et al.*, 1992; Mitaka *et al.*, 1994; Murray & Freeman, 1996). Also, various *in vitro* investigations have shown a direct negative inotropic action of TNF (Finkel *et al.*, 1992; Foulkes & Shaw, 1992; Yokoyama *et al.*, 1993; Weisensee *et al.*, 1993; Goldhaber *et al.*, 1996; Oral *et al.*, 1997). In addition to this, media from activated macrophages, containing cytokines, as well as mixtures of cytokines themselves have been shown to disrupt β-adrenergic function (Gulick *et al.*, 1988; Chung *et al.*, 1990; Balligand *et al.*, 1993b). All of these will be discussed in detail in section 1.5. So due to the increased circulating levels of TNF during shock, and the fact that TNF is a cardiodepressant in its own right, TNF has become a prime candidate as a mediator of the cardiac abnormalities observed during septic shock.

1.4 Heart disease and TNF

Chronic heart failure is a syndrome that has symptoms of fatigue and dyspnea and where impaired left ventricular function leads to many secondary changes in other organs. During the development of chronic heart failure the heart can undergo structural changes characterised by enlargement of the left-ventricular cavity, which is often accompanied by gross histological cardiac changes manifested by hypertrophy or even thinning of the ventricular wall, processes known as "remodelling". In addition to this, there are structural changes at the microscopic level which include increased collagen content of the extracellular matrix, which can cause a general "stiffening" of the ventricular wall and, therefore, impair relaxation. These changes in structure are accompanied by left ventricular dysfunction, and impaired ability to respond to the increased demands of exercise. The sensation of dyspnea, suggests alterations in pulmonary physiology, and indeed, pulmonary oedema is often observed (for review see Coats, 1997).

As heart failure progresses patients can develop a syndrome known as cardiac cachexia, characterised by profound weight loss and anorexia, which is generally accompanied by inflammation (Anker & Coats, 1997). The initial study implicating increased TNF levels in chronic heart failure (Levine *et al.*, 1990), showed that TNF levels were increased in patients with chronic heart failure, but this was limited to a subset of patients with cachexia. The ability of TNF to induce cachexia was described in section 1.2. Increased serum TNF in cachexic patients with heart failure has subsequently been shown on many other occasions (McMurry *et al.*, 1991; Dutka *et*

al., 1993; Katz *et al.*, 1994), and recently increased levels of TNF and its binding proteins have now been shown in heart failure without cachexia (Ferrari *et al.*, 1995).

Raised circulating TNF levels in chronic heart failure is reason enough to investigate the cardiac actions of TNF, however a review of the literature shows that many of the clinical hallmarks of heart failure can be mimicked by TNF, namely cardiac remodelling (Bozkurt *et al.*, 1998) and cardiomyopathy (Hegewisch *et al.*, 1990), left ventricular dysfunction (Pagani *et al.*, 1992) and pulmonary oedema (Millar *et al.*, 1989). Therefore the characterisation of the exact cardiac effects of TNF is important and could lead to novel therapeutic strategies in the treatment of chronic heart failure.

Two recent studies have investigated the effects of overexpression of TNF in the hearts of transgenic mice (Bryant *et al.*, 1997; Kubota *et al.*, 1997). The first of these showed biventricular dilation with a decreased ejection fraction, supporting a role for TNF in the pathogenesis of heart disease (Bryant *et al.*, 1997). In the second study, very similar results were obtained, where TNF overexpression lead to an increase in the heart:body weight ratio, with ventricular dilation and decreased ejection fraction (Kubota *et al.*, 1997). In addition to this it was shown that there was a decreased responsiveness to isoprenaline (Kubota *et al.*, 1997). β-adrenergic dysfunction is common in heart failure (Bristow, 1984).

Raised serum TNF concentrations have also been observed during ischaemic heart disease (Vaddi *et al.*, 1994), and a number of investigations have implicated TNF in the pathogenesis of experimental ischaemia. Squadrito *et al.* (1993) showed that in an anaesthetized rat model of coronary artery ligation, serum TNF levels were markedly

increased upon release of the coronary artery ligature. Immunisation with a hyperimmune serum containing antibodies against TNF showed a significantly increased survival rate for experimental animals (Squadrito *et al.*, 1993). During a study of hepatic ischaemia reperfusion, increased TNF was again observed upon reperfusion (Colletti *et al.*, 1993). Pulmonary injury was also observed during reperfusion, secondary to hepatic injury. Pre-treatment with anti-TNF antibodies effected complete protection from both the pulmonary oedema observed, and from hepatic injury seen. Very recently Meldrum *et al.* (1997) have shown that TNF is released from an *in vitro* perfused heart preparation after an ischaemic episode followed by reperfusion. Prior treatment with adenosine improved recovery of these hearts, as well as decreasing TNF release. Another interesting, very recent, study by the same group showed that TNF is expressed in the human myocardium following the obligatory global ischaemia during cardiopulmonary bypass (Meldrum *et al.*, 1998)

1.4.1 Cardiac acidosis

During cardiac ischaemia, cardiac acidosis can often be observed, indeed pH values of below 6.0 have been reported (Kolocassides *et al.*, 1996). This acidosis is the result of a build up of lactic acid from anaeobic glycolysis, as well as the release of protons from ATP hydrolysis. Interestingly, it has been reported that at low pH values, in the presence of TNF, ion channels can form in artificial membranes (Kagan *et al.*, 1992). Further investigation provided evidence that during low pH conditions, the compact trimer configuration of biologically active TNF was relaxed, allowing insertion into the plasma membrane (Baldwin *et al.*, 1996). This was accompanied by an increase in

sodium (Na^+) flux across the membrane. Elevated intracellular Na^+ can cause an increase in intracellular Ca^{2+} via the Na^+/Ca^{2+} exchanger (Tani & Neely, 1989). Therefore, during acidosis TNF could contribute to the Ca^{2+} overload seen during ischaemia by forming Na^+ permeable channels. Indeed, NMR experiments have shown that increased intracellular sodium has been is seen during ischaemia (Pike *et al.*, 1990).

Increases in serum TNF have also been observed in other cardiac diseased states including acute myocardial infarction (Latini *et al.*, 1994), cardiac allograft rejection (Arbustini *et al.*, 1991) and acute viral myocarditis (Smith & Allen, 1992).

1.5 Actions of TNF in the heart

Due to the aforementioned potential role for TNF as a mediator of the observed alterations in cardiac function during septic shock (section 1.3), as well as the marked increase in circulating TNF levels seen in various cardiovascular disease states (section 1.4), the direct cardiac actions of TNF have been a subject of increasing interest. Whilst it is generally acknowledged that TNF can disrupt normal heart function, much controversy still exists as to the precise nature of this disruption and many potential mechanisms have been suggested. There appear to be two phases in the observed depression in cardiac function produced by TNF, an early phase (< 30 min) and a late phase (> 120 min). In addition to this actions of TNF have been observed in the coronary circulation, independent of any cardiac depression. The following section will discuss the above observations in detail.

1.5.1 TNF induced immediate negative inotropic effects

Various *in vitro* and *in vivo* studies have described an early direct and reversible negative inotropic action of TNF (Finkel *et* al., 1992; Foulkes & Shaw, 1992; Weisensee *et al.*, 1993; Yokoyama *et al.*, 1993; Kapadia *et al.*, 1995; Torre-Amione *et al.*, 1995; Goldhaber *et* al., 1996; Oral *et al.*, 1997) however, others have disputed this early action of TNF (Mitikata *et* al., 1994; Schulz *et* al., 1995; Murray & Freeman, 1995; Nishikawa *et al.*, 1996). The studies by both Schulz and Murray & Freeman actually showed an initial positive inotropic effect of TNF.

In 1992, Finkel *et al.* were the first to show a direct and early negative inotropic action of TNF. This landmark paper describes studies in the electrically stimulated isolated ferret papillary muscle, where TNF caused a concentration-dependant decrease in contractile function, which was evident after 2 - 3 min, and reached a maximum after 5 min. Moreover these responses were shown to be reversible upon wash-out of TNF. This negative inotropic response was sensitive to NO synthase inhibitors, suggesting a vital role for NO (NO is briefly review in section 1.5.2). The rapid time-course required for this action implicated the constituatively expressed form of CO synthase (cNOS) as the enzyme responsible. In contrast to these observations, Yokoyama *et al.* (1993), showed that in the isolated feline heart as well as in isolated adult feline ventricular myocytes TNF caused an early, concentration-dependent and fully reversible negative inotropic effect which was insensitive to NO synthase and cyclooxygenase inhibition. The reversibility of this action and the lack of fragmented DNA suggested that responses seen were not secondary to necrotic or apoptotic cell death. An important observation of this study was that the negative inotropic effect was accompanied by a decline in the systolic peak intracellular calcium $[Ca^{2+}]_i$ transients. This was not attributable to changes in the voltage-sensitive calcium current (L-type Ca^{2+} current). The discrepancy between these two important investigations is not clear, species variability is one possible explanation, but this is not entirely satisfying.

In 1993, Weisensee *et al.* showed that within minutes TNF could cause complete cessation of the spontaneously beating rhythmic contractions seen in murine isolated neonatal myocytes, which with time progressed to arrhythmic beating, they also

showed that TNF caused a decrease in the resting membrane potential but the mechanisms responsible for these actions were not investigated.

In 1995 the same group which showed decreased $[Ca^{2+}]_I$, also demonstrated, using mutants of TNF which selectively bind to each TNF receptor, that the TNF type I receptor was responsible for the negative inotropic actions of TNF in adult rat cardiac ventricular myocytes (Torre-Amione *et al.*, 1995). Krown *et al.* (1995) presented data which partially supported the observations by Yokoyama *et al.* (1993) as well as those of Torre-Amione *et al.* (1995) showing that TNF could cause depressed $[Ca^{2+}]_i$ transients, but unlike Yokoyama *et al.* (1993), these other studies was concluded that inhibition of the L-type Ca^{2+} channel was likely to be the mechanism responsible. Single cell reverse transcription-PCR, [^{125}I]-TNF ligand binding and Western immunoblotting experiments again implicated the TNF type I receptor in these actions of TNF. However, this study did not include any data concerning the contractile activity of the cardiac myocytes involved.

Support for the publication by Finkel *et al.* (1992) implicating NO in the early negative inotropic actions of TNF was presented in a study using isolated adult rabbit ventricular myocytes (Goldhaber *et al.*, 1996). It was shown that, within 20 min, TNF could decrease cell shortening in response to electrical stimulation, an action which in contrast to Yokoyama *et al.* (1993) was not due to changes in $[Ca^{2+}]_i$ transients. Again this action was blocked by a NO synthase inhibitor (L-NAME), as well as by haemoglobin, a scavenger of NO. This suggests that a possible mechanism for the negative inotropic action of TNF involves the release of NO. It was suggested that this

NO could then cause decreased responsiveness of the cardiac myocyte myofilaments to Ca^{2+} (Goldhaber *et al.*, 1996).

In 1997, Oral *et al.* outlined a mechanism by which TNF could cause depressed $[Ca^{2+}]_i$ transients in cardiac myocytes via TNF-stimulated increases in free sphingosine. Both TNF and exogenous sphingosine caused an immediate (5 - 15 min) negative inotropic action. The actions of TNF could be blocked by an inhibitor of the enzyme responsible for the metabolism of sphingomyelin to sphingosine, N-oleoylethanolamine (NOE). This study was again conducted in isolated adult feline ventricular myocytes. A later study (Bozkurt *et al.*, 1998) subsequently showed that NOE could also partially attenuate the depression in cardiac function *in vivo* observed upon slow TNF infusion into rats.

As mentioned in section 1.1.5.3, sphingosine is an endogenous inhibitor of PKC, however this is not the only biological action of this sphingomyelin metabolite. In a study utilising skinned rabbit skeletal muscle fibres it was shown that sphingosine could act as a ligand for the ryanodine receptor (Sabbadini *et al.*, 1992). In this regard, sphingosine was shown to decrease caffeine-induced calcium release from internal stores. In line with this, sphingosine significantly increased the K_d for specific binding of [^{3}H]-ryanodine to terminal cisternae. This was in contrast to caffeine which increased K_d for ryanodine binding. In cardiac muscle, the ryanodine receptor is of great importance as it mediates a phenomenon known as calcium-induced calcium release (CICR). In cardiac myocytes depolarisation leads to relatively small amounts of Ca^{2+} entry through L-type calcium channels. This Ca^{2+} is thought to interact with the ryanodine receptor situated on the sarcoplasmic reticulum (SR), causing the release of

far greater quantities of Ca^{2+} from the SR into the cytosol, and this Ca^{2+} then causes a contraction (Fabiato, 1983). Soon after the initial report concerning the ryanodine receptor in skeletal muscle, it was shown that sphingosine could also disrupt the ryanodine receptor function in isolated canine cardiac SR membranes (Dettbarn *et al.*, 1994). Ca^{2+} release in response to caffeine, doxorubicin or by Ca^{2+} itself was also inhibited by prior administration of sphingosine. Sphingosine inhibited the extent of CICR, and significantly shifted the threshold for CICR, so that a higher level of trigger calcium was required. Another report from the same group showed that sphingosine could inhibit $[Ca^{2+}]_i$ transients in adult and neonatal cardiac myocytes, an effect which was dependant on a decrease in the L-type Ca^{2+} current as well as decreased CICR (McDonough *et al.*, 1994). Another report in the same year, by the same group showed that the disruptions in Ca^{2+} homeostasis seen upon sphingosine administration translated into depressed cardiac myocyte function (Webster *et al.*, 1994). These authors showed that sphingosine had no effect on the contractile machinery of the cardiac myocytes.

Thus, it is very plausible that sphingosine could mediate some of the early changes in cardiac function seen with TNF. However greater research is required in this area due to the reports suggesting a role for NO in this early depression in cardiac function. Neither of the two reports implicating NO in the early depression in function (Finkel *et al.*, 1992; Goldhaber *et al.*, 1996) convincingly demonstrated activation of cNOS which would be necessary for the rapid release of NO.

1.5.2 TNF induced late alterations in cardiac function

TNF induced late cardiac dysfunction has been observed on several occasions *in vivo* (Pagani *et al.*, 1992; Mitaka *et al.*, 1994; Murray & Freeman, 1996), and characterisation of this has proven more successful than investigations into the early depressant actions. Under *in vitro* conditions however, disruption in cardiac function almost always requires the addition of another cytokines such as IL-1. In the whole animal situation, TNF would be expected to cause the release of other cytokines including IL-1 (see section 1.1). Cytokine induced late cardiac dysfunction almost certainly involves NO synthesis, via an inducible form of NO synthase (iNOS), therefore a brief overview of the biology of NO will be given before presentation of the evidence supporting a role for NO in late contractile dysfunction seen with cytokines.

NO is a small lipophilic free radical gas which is synthesised from L-arginine and molecular oxygen, and has been shown to be identical the endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980). The synthesis of NO is dependent on a family of enzymes known as NO synthase (NOS) enzymes, all of which require NADPH, FAD, FMN, tetrahydrobiopterin and calmodulin as cofactors. The NOS family includes a constituatively expressed, Ca^{2+}-dependant (cNOS) as well as an inducible, Ca^{2+}-independent NO synthase (iNOS). Traditionally cNOS was thought to be expressed exclusively in the vascular endothelium, but it is now known that, amongst other cells, cardiac myocytes can also express cNOS (Balligand *et al.*, 1993a). cNOS generally produces NO in small "puffs". iNOS on the other hand is a high output source of NO which, with its tightly bound calmodulin, does not depend on increases in Ca^{2+}. iNOS can be induced by cytokines (Koide *et al.*, 1993), and this requires protein synthesis

and, therefore, may take several hours to become fully active (Tsujino *et al.*, 1994). A major target for the actions of NO is guanylate cyclase, which when activated increases the production of cGMP (Palacios *et al.*, 1989). However, NO can also react with the free radical superoxide anion (O_2^-), to form the highly reactive and potentially damaging molecule, peroxynitrite ($ONOO^-$) (Radi *et al.*, 1991).

One of the first studies to try to characterise a mechanism for the late cardiac dysfunction came from Gulick *et al.* (1988), who showed that in cardiac myocytes, several hours incubation with cell free supernatants from activated macrophages could inhibit the expected increase in cAMP, as well as the concomitant positive inotropic effects of isoprenaline. Supernatants from activated macrophage would be expected to contain many cytokines, especially TNF and IL-1. Another study from the same laboratory subsequently showed that supernatants from rat activated splenocyte cultures (containing TNF, IL-1 and IL-2), could also cause inhibition of isoprenaline induced increases in cAMP in cardiac myocytes after 48-72 hours exposure (Chung *et al.*, 1990). This action was not altered by phosphodiesterase inhibitors or cholera toxin, but was inhibitable by petussis toxin, thus implicating altered coupling of β-adrenergic receptors to adenylate cyclase at the level of the stimulatory/inhibitory G-proteins (Chung *et al.*, 1990).

These studies were supported and extended by Balligand *et al.* (1993b), who showed that supernatant from LPS-activated macrophages, when incubated for 24 hours with cardiac myocytes could block the β-adrenergic receptor mediated inotropic response. Again this response was sensitive to pertussis toxin. Also shown in this study was the generation of NO with concurrent generation of cGMP. L-NMMA an inhibitor of all

isoforms of NOS could block the inhibitory effects seen on addition of isoprenaline. A subsequent study by the same group showed that exogenous cytokines could mimic these actions by induction of the iNOS enzyme, and an increased iNOS mRNA was observed after 12 hours (Balligand *et al.*, 1994). Cardiac cells other than myocytes may also be important in the hearts response to cytokines. In a study by Ungreau-Longrois *et al.* (1995) cardiac microvascular endothelial cells were cultured and treated with IL-1β for 24 hours and this induced iNOS in these cells. Following this, cardiac myocytes were allowed to settle onto the confluent layer of treated endothelial cells. Isoprenaline induced responses were reduced in myocytes when in this heterotypic culture. In the presence of L-NMMA, no such decline in isoprenaline response was seen. Thus, diffusion of NO from the endothelial cells was able to cause the subsequent blockade of responses in adjacent cardiac myocytes (Ungreau-Longrois *et al.*, 1995).

Evidence for a direct negative inotropic action involving cytokine induced induction of iNOS in the absence of either circulating catacholamines, or exogenously administered β-receptor agonists was presented in the isolated perfused rat heart. A combination of TNF and IL-1β could depress cardiac function only 2 hours after cytokine application (Schulz *et al.*, 1995). This depression in cardiac function did not require isoprenalinee to become apparent. No early negative inotropic effect was observed. The action of these cytokines could be blocked by the protein synthesis inhibitor cyclohexamide. Indeed, iNOS activity was evident, and the depression in cardiac contractility could be alleviated by NOS inhibition (Schulz *et al.*, 1995).

In summary, it is apparent that induction of iNOS over the period of several hours can lead to contractile dysfunction either by a direct NO-mediated negative inotropic action, or by a reduced responsiveness to β-receptor agonists. The precise mechanism behind these actions is not clear at present. Direct negative inotropic actions of NO have also been observed in the absence of cytokine stimulated iNOS expression (Smith *et al.*, 1991; Brady *et al.*, 1993). The negative inotropic effect of NO could be mediated by stimulation of guanylate cyclase (Mery *et al.*, 1993) or by direct inhibitory actions of NO on mitochondrial respiratory enzymes (Geng *et al.*, 1992). As mentioned in section 1.5.1 Goldhaber *et al.* (1996) presented evidence that NO could decrease Ca^{2+} responsiveness of myofilaments. The mechanism behind the apparent alteration in adenylate cyclase stimulation by β-adrenergic agonists upon cytokine incubation is also not clear at this point in time. Increased levels of cGMP, from guanylate cyclase stimulation by NO, could activate a cGMP-dependant cAMP phosphodiesterase, which in turn could breakdown the product of adenylate cyclase activation, cAMP, thereby reducing the effect of adenylate cyclase activation. However the studies by Chung *et al.* (1990), suggest that this is unlikely because the inhibition of isoprenaline-induced cAMP accumulation was not altered by a phosphdiesterase inhibitor. Instead the mechanism behind this action appears to involve a NO induced "uncoupling" of adenylate cylase from the β-adrenergic receptor, and studies with pertussis toxin implicate a role of the inhibitory G-protein in this action (Chung *et al.*, 1990; Balligand *et al.*, 1993b).

NO mediated depression in function is also, at least in part responsible for the contractile dysfunction observed during shock, as cardiac myocytes isolated from endotoxin treated guinea-pigs show depressed contractile function which is inhibited

by NOS inhibition (Brady *et al.*, 1992). In addition to this, NOS inhibition can partially prevent the decrease in left ventricular contractility observed upon *in vivo* administration of endotoxin (Herbertson *et al.*, 1996).

1.5.3 Other cardiac effects of TNF

As mentioned earlier (section 1.3.2) administration of TNF or LPS to whole animals results in constriction of the coronary circulation which is observed *ex vivo* when hearts were perfused by the Langendorff technique (Hohlfeld *et al.*, 1995). It was shown that administration of TNF or LPS caused increased circulating levels of ET-1, and that the coronary constriction could be blocked by an antagonist of the endothelin$_A$ (ET_A) receptor. In other studies from the same group it was shown that antibodies against ET-1 could ameliorate this *ex vivo* coronary constriction induced by TNF, and that exogenous administration of interleukin-2 caused release of TNF, which again induced an ET-1 mediated coronary constriction (Klemm *et al.*, 1995a). A subsequent investigation showed that during adjuvant-induced arthritis, increased circulating cytokine levels were observed (Klemm *et al.*, 1995b). When hearts were perfused from this arthritis model, again an increase in coronary tone was observed which was blocked by an endothelin receptor antagonist. The source of the ET-1 responsible for this constriction was not identified. If this coronary constriction occurred in the whole animal situation, a decrease in substrate supply to the heart could mediate an indirect depression in cardiac function.

1.6 Aims of this study

1) To investigate the effects of recombinant human TNF on insulin action in the isolated perfused rat heart under substrate limiting conditions, i.e. low glucose perfusion and low flow ischaemic perfusion. Under such conditions, the actions of insulin would be expected to be exacerbated. To attempt to characterise any TNF mediated disruptions in insulin action with the use of pharmacological agents.

2) To investigate cardiac depressant actions of TNF in the isolated perfused rat heart, utilising left ventricular developed pressure, rate of change of both systolic contraction and diastolic dilation, heart rate and Starling curves to assess cardiac function. To attempt to characterise any depression in function seen with pharmacological agents.

3) To see if TNF has any direct actions on the coronary circulation of the rat heart, and to attempt to characterise any action seen. Also to see if any action of TNF in the coronary circulation can affect cardiac function.

4) To observe the recovery of hearts after short periods of acidosis, and to see if TNF adversely affects this recovery.

Section 2 Materials and Methods

2.1 Materials

British Drug House (BDH), Poole: D-glucose, ethanol (96%), ethylenediaminetetra-acetic acid (EDTA), glycoge (oyster), KCl, NaCl, $NaHCO_3$.

Biomol, Affiniti Research Products Ltd., Exeter: Okadaic acid

Fisons Scientific Equipment, Loughborough: $CaCl.6H_2O$, Hydrazinium sulphate, KH_2PO_4, $MgSO_4$, $MgCl_2.6H_2O$, NaOH,

Peninsula Laboratories, Merseyside: endothelin-1

R&D systems, Abingdon: recombinant rat TNF-α

Sigma, Poole: adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), β-nicotinamide adenine dinucleotide (NAD^+), β-nicotinamide adenine dinucleotide phosphate (NADP), D-sphingosine, fructose 6-phosphate (F-6-P), fructose 1,6-bisphosphate (F-1,6-P), glucose 1-phosphate (G-1-P), glucose 6-phosphate (G-6-P), glycine, indomethacin, L-lactate, N-acetly-$_D$-sphingosine (C_2-ceramide) Nω-nitro-l-arginine, N-oleoylethanolamine, phosphocreatine (PCr), U46619 (9,11-dideoxy-9α,11α-epoxy-methanoprostaglandin $F_{2\alpha}$).

Enzymes purchased from **Sigma**, Poole: aldolase (type IV, from rabbit muscle), creatine kinase (type I from rabbit muscle), glucose 6-phosphate dehydrogenase (type VII from bakers yeast), glycerol-3-phosphate dehydrogenase (type I from rabbit muscle), hexokinase (type C-130 from bakers yeast), lactate dehydrogenase (type I from bakers yeast), phosphoglucose isomerase (from bakers yeast), phosphoglucomutase (from rabbit muscle), sphingomyelinase (SMase, from *Bacillus Cereus*), triosephosphate isomerase (type I from bakers yeast).

ZD1542 (4(z)-6-[2S,4S,5R]-2-[1-methyl-1-(2-nitro-4-tolyloxy)ethyl]-4-(3-pyridyl0-1,3-dioxan-5-yl]hex-4-enoic acid) was a gift from Dr. M.J. Wayne (Zenica). GR32191 (([1R-(α(Z),2β,3β,5α]]-(+)-7-[5-([1,1'-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl)-cyclopentyl]-4 heptonoic acid) was a gift from Glaxo Pharmaceuticals. Bosentan was a kind gift from Dr. A. G. Roach (Rhone-Poulenc Rorer, Dagenham). Recombinant human TNFα was a very kind gift from Bayer (Slough).

Male Wistar rats, University of Bath strain, 280-310g have been used throughout this project. In all experiments animals were anaesthetised by an interperitoneal injection of sodium pentobarbitone (200 $mg.kg^{-1}$), and then killed by cervical dislocation.

2.2 The Langendorff isolated perfused heart

The heart of each animal was excised and perfused using a modified Langendorff technique via a cannula inserted in the aorta, care was taken not to disrupt the aortic valves during this process. Hearts were perfused under conditions of constant flow (10 $ml.min^{-1}$ or 2 $ml.min^{-1}$) or constant pressure conditions (70 mmHg) with prefiltered, oxygenated (O_2 95%, CO_2 5%) Krebs-Henseleit solution, at 37°C (unless otherwise stated), of the following composition:

NaCl	118 mM
$NaHCO_3$	25 mM
KCl	4.7 mM
KH_2PO_4	1.2 mM
$MgSO_4.\ 7H_2O$	1.2 mM
$CaCl_2$	1.2 mM
D-glucose	11.6 mM

In some experiments glucose levels were altered, and concentrations of both 2 mM and 25 mM were used. In other experiments $NaHCO_3$ concentrations were lowered in order to alter the pH of the Krebs buffer to either pH 6.1 or pH 5.5. Hearts were

allowed to beat spontaneously throughout all experimental procedures (Langendorff, 1895).

A recirculating perfusion method was chosen to examine the cardiac actions of TNF. The major advantage of this system is that small volumes of perfusate are required, decreasing the amount of any drug used. A disadvantage of this system is that, once added, drugs cannot be washed out easily without disrupting the recirculating process. A total volume of 50 ml was chosen for recirculation.

2.2.1 Parameters measured

Under constant flow conditions, coronary perfusion pressure (CPP) was measured using a Gould P231D pressure transducer, and used as a measure of coronary tone. In hearts perfused under a constant head of pressure, coronary inflow was measured using a T 206 Transonic Systems Inc. flow meter, attached to the aortic cannula, and used as a measure of coronary tone. Contractile function of hearts was measured using two different methods, one utilising a hook placed in the apex of the heart, and another using intraventricular balloons, these are both described in detail below.

When using the hook method, then developed tension and heart rate were used as a measure of cardiac function, and were recorded via a hook in the apex of the heart, connected by a pulley system to an isometric force transducer (Dynometer UFI). Each heart was initially placed under a resting tension of 2 g. A small gauge needle (0.5 x 16 mm) was inserted into the left ventricle to allow Thebesian drainage, this is necessary in order to drain the left ventricle of its venous return (figure 2.1).

The other method for measuring heart function utilised an intraventricular balloon. Hearts were prepared as before, and a fluid filled Clingfilm balloon was inserted into the left ventricle and inflated (great care was taken to ensure the absence of air bubbles within the balloon). Pressure changes inside the balloon were measured via a Gould P231D pressure transducer and left ventricular developed pressure (LVDP) and heart rate were used as in index of cardiac function. In addition to this, the signal generated from the balloon was differentiated by a Grass polygraph differentiation, 7P20C, in order to measure the rate of change of systolic contraction and rate of change of diastolic relaxation. Left ventricular-end diastolic pressure (LVEDP) was set between 5 and 10 mmHg with the use of a 1 ml syringe connected to a micrometer head, allowing fine adjustment of balloon volume. The advantage to using an intraventricular balloon is that Starling curves can be performed to asses the Frank-Starling response in perfused hearts. In these, studies Starling curves were performed by emptying the intraventricular balloon, then slowly filling the balloon, to a total volume of 0.24 ml, in 0.03 ml aliquots every 30 seconds. LVDP was then measured and plotted against the volume added to the balloon. This results in very reproducible volume-response curves which represent the hearts ability to respond to changes in diastolic volume. Output from all transducers was recorded on either a Grass model 7B polygraph, a Gould 2400S chart recorder, a Gould RS3400 chart recorder or on a Macintosh Performa 5400/160 with the use of Maclab/4e.

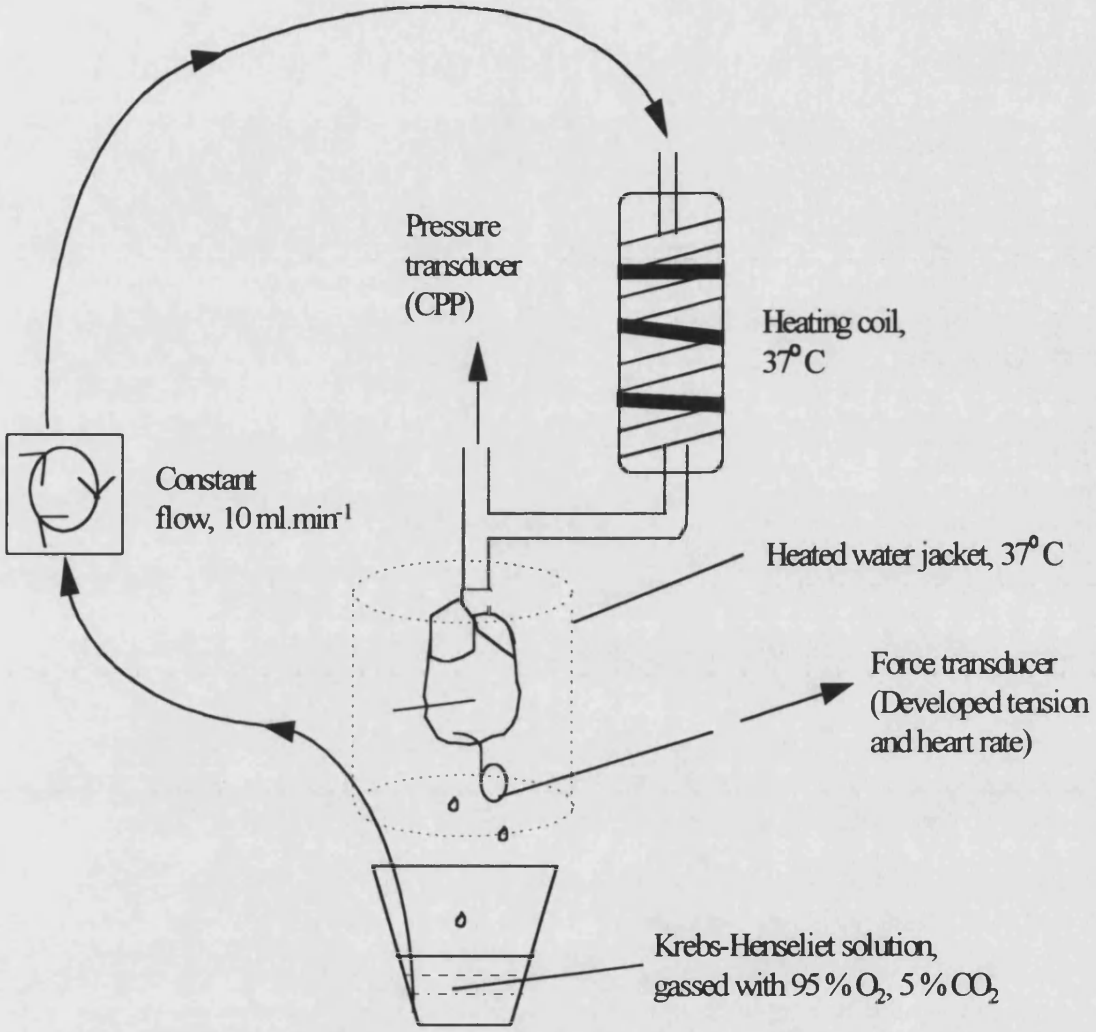


Figure 2.1. A diagramatical representation of the recirculating perfusion set up used in all preliminary experiments.

2.3 Protocol for assays used

Throughout this study assays have been used to measure glucose uptake from and lactate accumulation in the recirculating Krebs buffer. In addition to this, further assays have been completed in order to determine the heart tissue levels of glycogen, glucose 6-phosphate (G-6-P), glucose 1-phosphate (G-1-P), adenosine triphosphate (ATP), phosphocreatine (PCr), fructose 6-phosphate (F 6-P) and fructose 1,6-bisphosphate (F-1,6-P). All of these are described below.

2.3.1 Assay for glucose

The principle behind the assay used to measure glucose levels, in samples from the recirculating perfusate, was dependant on the following reaction:

$$\text{(1)}\quad \text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{(2)}\quad \text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-Phosphogluconate} + \text{NADPH}$$

HK = Hexokinase

G-6-PDH = Glucose-6-phosphate dehydrogenase

(Stein, 1963)

The reaction mixture for this assay was of the following composition: $NADP^+$ 1.5 mM, ATP 1 mM, MgCl 2.1 mM, HK 1000 $U.l^{-1}$ and G-6-PDH 1000 $U.l^{-1}$, all quantities being in excess. To 1 ml of this solution was added 50 µl of the sample to be assayed, this was then left to stand for 5 min at room temperature. 50 µl of distilled water was used as the control. The absorbance of NADPH in the solution was measured in a quartz cuvette at 340 nm and the concentration of glucose calculated using a calibration curve constructed from glucose standards (0.1 mM, 0.2 mM, 0.4m M, 0.8 mM, 1.6 mM and 3.2 mM).

2.3.2 Assay for lactate

The spectrophotometric determination of lactate concentration is dependant on the following reaction:

$$\text{(3) L-lactate} + NAD^+ + \text{Hydrazine} \xrightarrow[\text{pH 9.5}]{\text{LDH}} \text{Pyruvate Hydrazone} + NADH + H_3O^+$$

LDH = lactate dehydrogenase

Shown below is the reaction mixture, added to the cuvette, and required for the determination of lactate within a given sample:

Hydrazone (0.4 M) - glycine (0.5 M) buffer (pH 9.5), with 20 mM EDTA	0.625 ml
NAD^+ solution, 0.045 M	0.05 ml
Lactate dehydrogenase, 5000U/ml	5 µl
Sample to be assayed or blank	0.05 ml

After 30 min incubation at 37°C, NADH was measured spectrophotometricaly at 340 nm, and concentration of lactate calculated using the extinction coefficient of NADH at 340 nm, whereby a solution containing 1 mM NADH would have an absorbance constant of 6.22, when measured at 340 nm. This value is stoichiometrically related to the lactate concentration. The method of lactate determination has been described fully by Hohorst, 1965.

2.3.3 Glycogen determination

Hearts were freeze clamped, between a pair of Wollenberger tongs which had been cooled to the temperature of liquid nitrogen (-196°C), the resultant disc of tissue was then stored at -80°C until such time as an assay of the levels of glycogen could be undertaken.

Glycogen levels were determined by hydrolysis to glucose and subsequent calculation of glucose concentration as described previously (Pfleiderer, 1965). The frozen hearts were powdered using an homogeniser, previously cooled in liquid nitrogen. The finely powdered tissue, approximately 250 mg, was placed in a pre- weighed test tube containing 2 ml KOH (30 % w/v) and 2.8 ml absolute ethanol (95%). The test tube was then reweighed to determine tissue weight, then placed in a boiling water bath for 30 min to allow alkaline digestion of the tissue followed by glycogen extraction with ethanol. Tubes were centrifuged for twenty minutes at 1250g, the resulting supernatant was discarded and the pellet washed in 5 ml of 70 % v/v ethanol. The tubes were then recentrifuged, the supernatant was again discarded and the pellet dried at 100°C. 1 ml of 2M sulphuric acid was added to each tube, the tubes were placed in a boiling water bath, stoppered and then left for two hours. The acidic solution was neutralised using 2M sodium hydroxide and to this was added 5 ml of phosphate buffer (pH 7.5), and total volume measured. Concentration of glucose could then be determined using the method described previously, 2.3.1. Glycogen levels were expressed as μmoles of glucose equivalents per gram of tissue, dry weight.

2.3.4 Glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, ATP and PCr determination

Glucose 6-phosphate (G-6-P, glucose 1-phosphate (G-1-P), fructose 6-phosphate (F-6-P), adenosine triphosphate (ATP) and phosphocreatine (PCr) were all measured using the same extraction procedure. The reactions which this depends on are shown in figure 2.2. Each was then determined within the same cuvette as described below. Freeze clamped hearts were powdered as described above. Approximately 150 mg of frozen ventricular tissue was added to a preweighed 1.5 ml centrifuge tube containing 1 ml ice-cold perchloric acid (6% w/v). This was then reweighed to determine the exact tissue weight. The samples were left on ice to extract for 30 min, with regular mixing intervals. 2 min centrifugation, at 12000g, resulted in a distinct supernatant, 500µl of which was removed and neutralised with KOH (40 % w/v) using 5µl of universal indicator. This was then allowed to stand on ice for 5 min, whereupon a sediment of $KClO_4$ was formed, after decanting the solution, it was taken immediately for analysis of G-6-P, G-1-P, F-1-P, ATP and PCr.

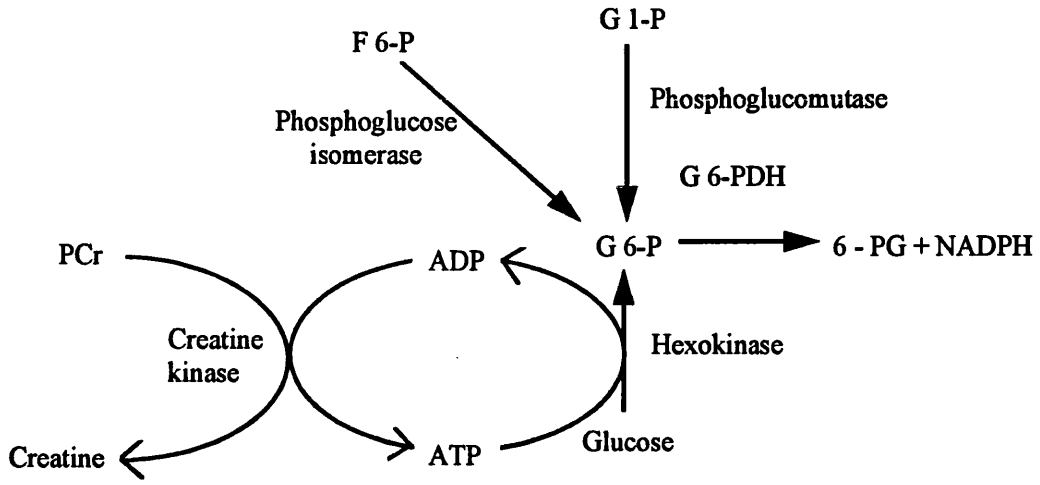


Figure 2.2. A diagrammatical representation of the reactions involved in the determination of G 6-P, G 1-P, F 6-P, ATP and PCr. 6-PG = 6-phosphogluconate.

The reaction utilised by this assay follow the same principle as that for glucose determination except, stepwise addition of different enzymes allowed determination each substrate to be measured. The reaction mixture for this assay is as follows:

Distilled water	550 µl
Phosphate buffer, pH 7.5	350 µl
$NADP^+$, 13 mM	35 µl
Glucose, 100 mM	20 µl
$MgCl_2$, 1 M	35 µl
ADP, 35 mM	8 µl

To 1 ml of this solution was added 40 µl of the sample to be assayed. Absorbance was determined, after which 2 µl of G-6-PDH (1.1U), was added and the following change in absorbance measured, after 5 min, and resulted from reaction (2). After this addition of 5 µl HK (2.8 U) allowed reaction (3) to take place and determination of ATP via G-6-P can be calculated. After the absorbance reading stabilised, about 5 min, the value was recorded. Addition of creatine kinase (CK), 5 µl (2.8 U), allowed reaction (4) to take place, and so determination of PCr was possible.

$$(4)\ \text{PCr} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP}$$

Therefore the generation of more ATP allows reactions 1 and 2 to begin again. After a further period to allow the reaction to run to completion, 2 µl of phosphoglucomutase (3.13 U) allowed reaction (5) to proceed.

$$\text{(5) G-1-P} \xrightarrow{\text{phosphoglucomutase}} \text{G-6-P}$$

When the absorbance reached a maximum, 2µl of phosphoglucose isomerase (4U) was added to the reaction mixture in order to measure F-6-P via reaction (6).

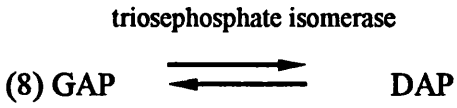
$$\text{(6) F-6-P} \underset{\text{phosphoglucose isomerase}}{\rightleftharpoons} \text{G-6-P}$$

Therefore, measurement of $NADP^+$ spectrophotometrically allowed estimation of G-6-P, G-1-P, F-1-P, ATP and PCr extracted from hearts using a calibration curves constructed from standards. The calibration curves used the following standards, prepared from each of the substrates to be measured: 0.1 mM, 0.25 mM, 0.5 mM and 1 mM. Levels are expressed as µmoles per gram dry weight (Lamprechet & Trantschold, 1974, Lamprechet, *et al.* 1974).

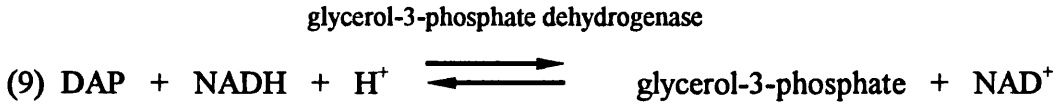
2.3.5 Determination of tissue fructose 1,6-bisphosphate levels

When determining tissue levels of fructose 1,6-bisphosphate (F-1,6-P), after extraction of F-1,6-P, one must first split the molecule to dihydroxyacetone phosphate (DAP) and glyceraldehyde 3-phosphate by reaction (7).

GAP must then be converted to DAP by reaction (8).



DAP can then be converted to glycerol-3-phosphate according to reaction (9), and then NAD^+ levels can be determined spectrophotometrically.



Freeze clamped hearts were powdered as described above. Approximately 300 mg of frozen ventricular tissue was added to a preweighed centrifuge tubes containing 5 ml perchloric acid, 0.6 M. Tubes were then re-weighed to determine the exact tissue weight. After 10 min centrifugation at 3000 rpm, the supernatant was extracted, and the sediment mixed with 1 ml perchloric acid and 1 ml distilled water. This was then centrifuged again for 10 min at 3000 rpm. After this, the two supernatants were combined and the pH adjusted to 3.5 with 5 M potassium carbonate solution. This was then brought to a final volume of 8 ml and allowed to stand on ice for 15 min, after which the supernatant was pipetted off, and portions of this used in the assay mixture.

The following was added to a quartz cuvette:

Buffer (triethanolamine hydrochloride, 0.4 M, pH 7.6; EDTA 40 mM)	0.5 ml
Supernatant from extraction procedure	0.5 ml
NADH solution (5 mM)	3 µl
Glycerol-3-phosphate dehydrogenase (130 $U.l^{-1}$)	3 µl
Triosephosphate isomerase (830 $U.l^{-1}$)	3 µl

This solution was then left in the cuvette until the absorbance reading had stabalised, measured at 340 nm. This was to allow the conversion of GAP and DAP extracted from the heart tissue to be completely converted to glycerol-3-phosphate. Aldolase, 3 µl, 45 $U.l^{-1}$, to the cuvette, and the absorbance change measured. F-1,6-P_2 levels were expressed as µmoles per gram of tissue, dry weight. This assay procedure is described fully by Slater, 1953.

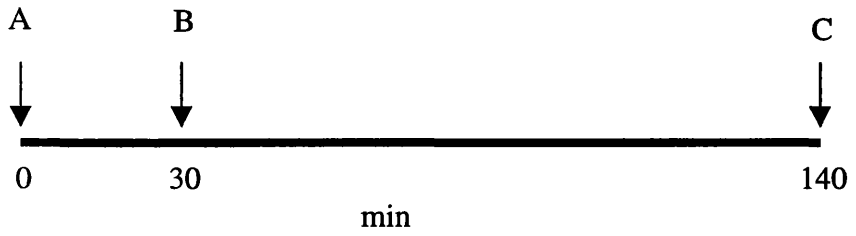
2.3.6 Determination of wet weight/dry weight ratio

A small piece of ventricular tissue was taken and excess Krebs buffer removed by blotting. The tissue was then placed in a 1.5 ml centrifuge tube of known weight, the total weight was subsequently determined. This was then placed in a vacuum oven overnight at 80°C, before being removed and cooled to room temperature. The dry weight of the tissue was then determined and a ratio between dry weight and wet weight was calculated.

2.4 Protocols for isolated heart perfusion

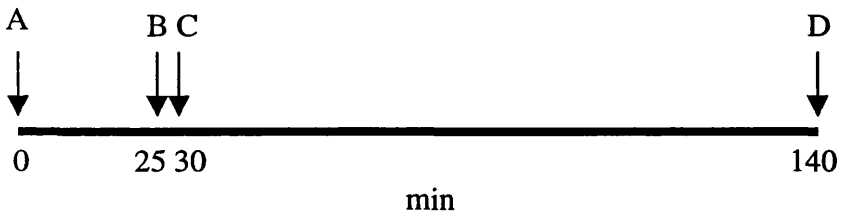
Shown below are diagrammatical representations of the protocols followed in this study. These are repeated during the results section:

A)



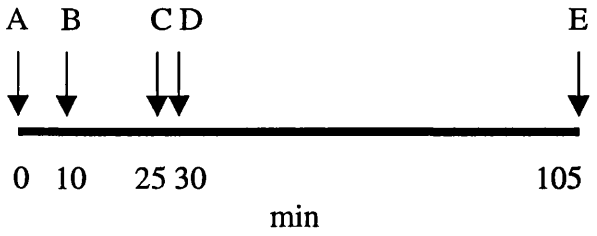
A = initial perfusion, B = recirculation, C = end of perfusion

B)



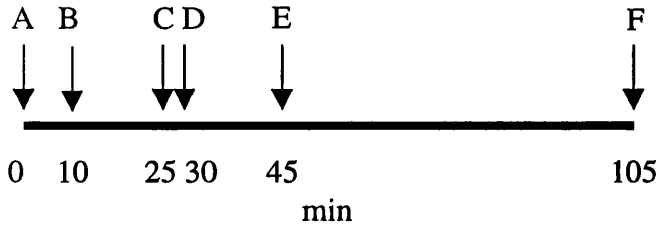
A = initial perfusion, B = TNF, 20 $ng.ml^{-1}$, C = recirculation,
D = end of perfusion

C)



A = initial perfusion, and addition of insulin, 10 $U.l^{-1}$, B = Low glucose, 2mM,
C = TNF, 20 $ng.ml^{-1}$, or C_2-ceramide, 1 μM, 5 μM or 10 μM, D = recirculation,
E = end of perfusion, freeze clamp TNF treated hearts

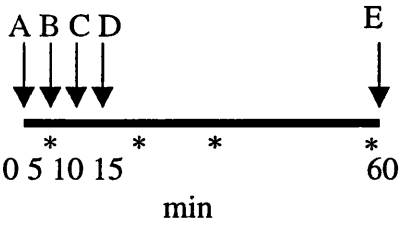
D)



A = initial perfusion, B = Low glucose, 2mM, C = TNF, 20 ng.ml^{-1},

D = recirculation, E = insulin, 10 U.l^{-1}, F = end of perfusion, freeze clamp hearts

E)

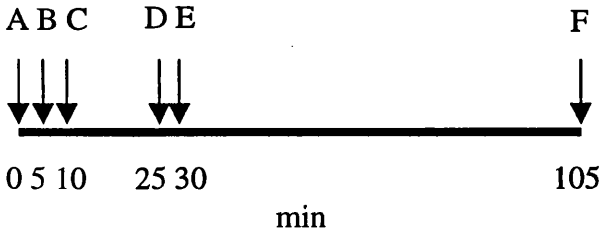


A = initial perfusion, B = zero glucose, C = TNF, 20 ng.ml^{-1}, D = recirculate,

E = end of perfusion.

* show the various points in the experiment where hearts were freeze clamped

F)



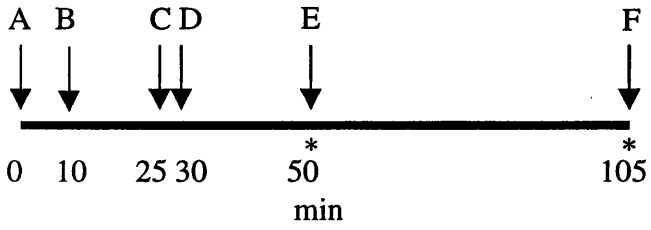
i) A = initial perfusion, and addition of insulin, 10 U.l^{-1} B = okadaic acid,

0.1nM or 1nM, C = Low glucose, 2mM, D = ceramide, 1μM, 5μM or 10μM,

E = recirculation, F = end of perfusion, freeze clamp hearts.

ii) B = okadaic acid, 1nM, D = TNF 20 ng.ml^{-1},

G)

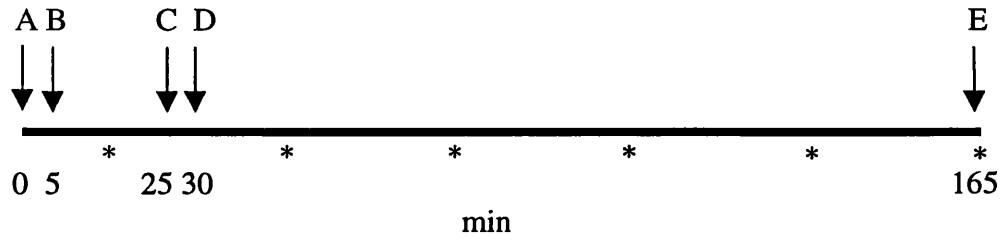


A = initial perfusion, B = Low glucose, 2mM, C = TNF, 20 ng.ml^{-1}, D = recirculation,

E = Low flow ischaemia, 2 ml.min^{-1}, F = end of perfusion.

* show the two points in the experiment where hearts were freeze clamped.

H)

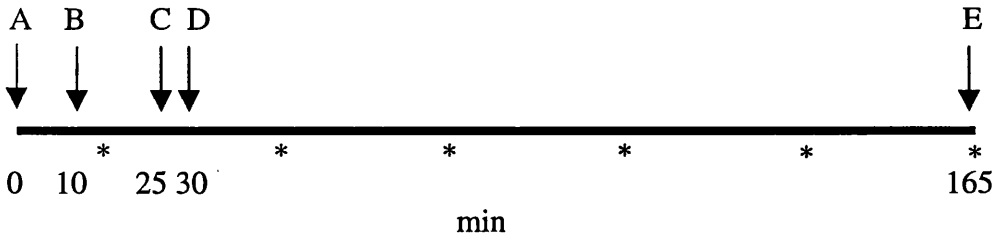


A = initial perfusion, B = nitro-l-arginine, 100 μM, C = TNF, 20 ng.ml^{-1},

D = recirculation, E = end of perfusion, hearts freeze clamped.

* show where Starling curves were performed

I)

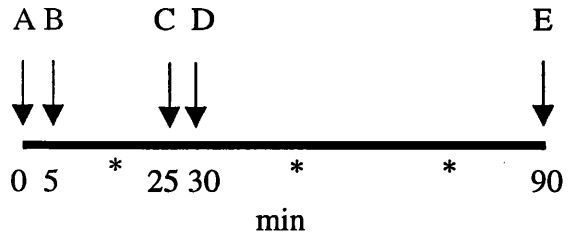


A = initial perfusion, B = high glucose perfusion, 25 mM, C = TNF, 20 ng.ml^{-1},

D = recirculation, E = end of perfusion, hearts freeze clamped.

* show where Starling curves were performed

J)

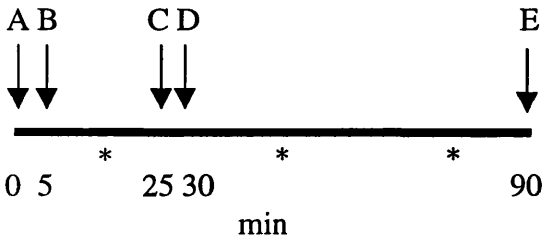


A = initial perfusion B = antagonist or inhibitor C = recombinant human TNF or recombinant rat TNF, 20 ng.ml^{-1} or SMase, 0.001 U.l^{-1}, 0.003 U.l^{-1} or 0.01 U.l^{-1}

D = recirculation E = end of perfusion

* show where Starling curves were performed.

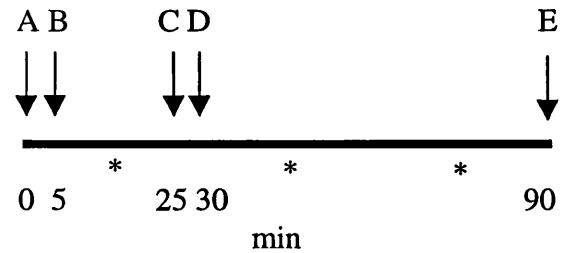
K)



A = perfusion under a constant head of pressure, B = NOE, 1μM, C = TNF 20 ng.ml^{-1}, D = recirculation, E = end of perfusion.

* show where Starling curves were performed.

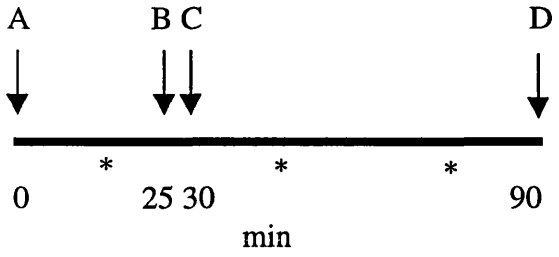
L)



A = initial perfusion, B = antagonists, C = sphingosine, 0.5 μM, 1 μM, 3μM or 10 μM, D = recirculation, E = end of perfusion.

* show where Starling curves were performed.

M)

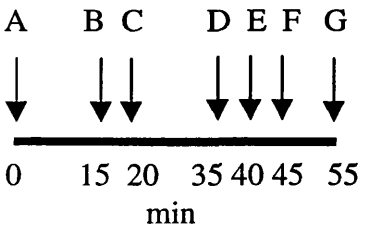


A = initial perfusion, B = sphingosine, 0.5 μM with TNF, 20 ng.ml^{-1},

C = recirculation, D = end of perfusion.

* show where Starling curves were performed.

N)



A = initial perfusion, B = acidosis C = recovery with normal pH,

D = TNF, 20 ng.ml^{-1}or no treatment, E = application of acidosis,

F = recovery with normal pH, G = end of perfusion.

2.5 Statistical analysis of data

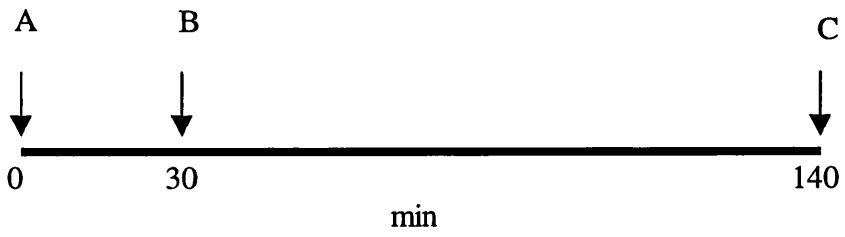
All values presented herein are shown as the mean ± standard error of the mean. Un-paired Student's t-test was used to compare differences between two grouped means from different data sets. Paired Student's t-test was used to compare differences between paired group means. Comparison of a control group mean with multiple test group means was conducted with one way ANOVA coupled to Dunnetts' post-hoc test for significance. Multiple comparisons between many group means was conducted using one way ANOVA coupled to Tukey's post-hoc test for significance. Comparison of all non-parametric data were conducted using Mann-Whitney test for significance. Statistical significance was accepted, and the null hypothesis was rejected, when $P < 0.05$, although when achieved, higher significance levels are also shown in this report.

Section 3 Results

3.1 Preliminary Experiments

3.1.1 Functional studies with TNF

Preliminary experiments were conducted in order to define the conditions under which to examine the actions of TNF. Hearts were perfused under constant flow conditions, 10 $ml.min^{-1}$. Cardiac contractility was measured using the hook method, where developed tension was used as a measure of cardiac function. Experiments were conducted in order to asses the viability of the isolated perfused heart under recirculating conditions. This was done by a direct comparison between hearts under recirculating and non-recirculating conditions over a time course of 140 min (figure 3.1) and followed the protocol shown:



A = initial perfusion, B = recirculation, C = end of perfusion

It was concluded from this that the recirculating heart, with a total volume of 50 ml was a viable model with which to examine the actions of TNF.

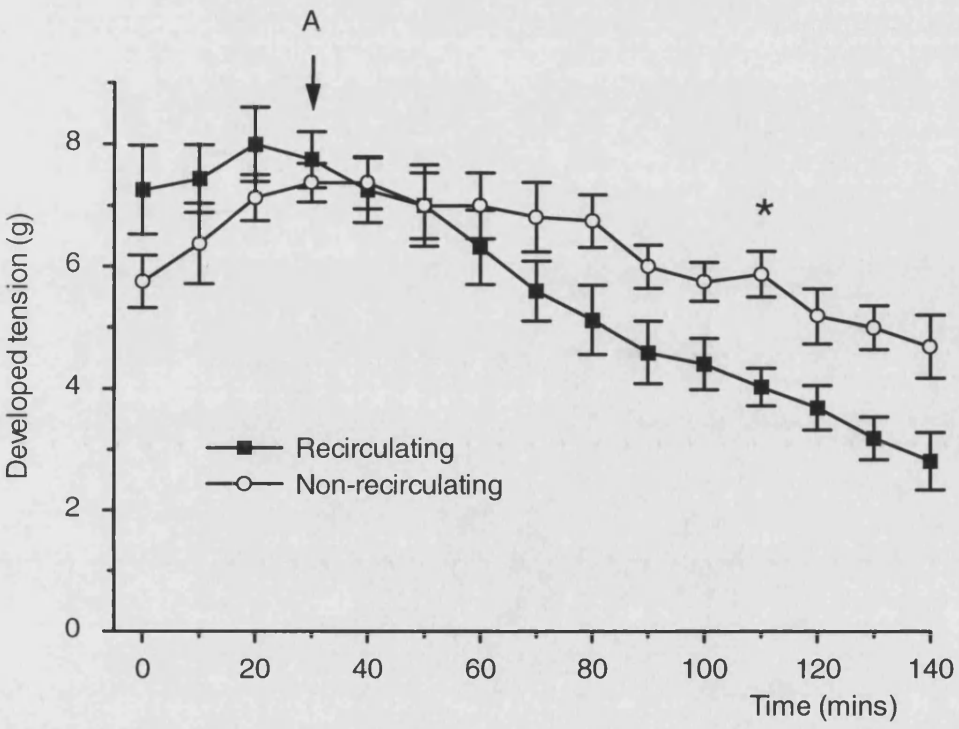
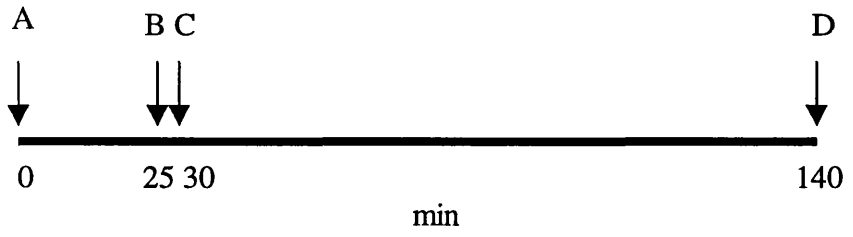


Figure 3.1. Effect of time on developed tension using recirculating (n = 8) and non-recirculating (n = 4) modes of perfusion in the isolated rat heart. Recirculation is initiated at A. * $P < 0.05$, for recirculating vs. non-recirculating.

Preliminary work was undertaken with TNF. Recombinant human TNF, 20 $ng.ml^{-1}$ and 100 $ng.ml^{-1}$, was added to the Krebs buffer after 25 min, 5 min prior to recirculation. 20 $ng.ml^{-1}$ TNF has been used in other experiments investigating the cardiac actions of TNF (Schultz *et al.*, 1995), and is a concentration which has been observed in sera from experimental models of septic shock (Tracey *et al.*, 1996). Perfusion continued for a further 110 min as shown below:



A = initial perfusion, B = TNF, 20 $ng.ml^{-1}$, C = recirculation,
D = end of perfusion

It can be seen from figure 3.2 that addition of TNF, 20 $ng.ml^{-1}$, caused a decline in function which was apparent within 15 min of TNF addition. It can also be seen that TNF, 100 $ng.ml^{-1}$, did not cause a greater depression in function. In all subsequent experiments TNF at the concentration of 20 $ng.ml^{-1}$ was used.

3.1.2 Extraction efficiencies of assays used

Experiments were conducted in order to discover the extraction efficiencies of each of the assays used. The efficiencies of each assay were as follows: G-6-P, 96 ± 3 %; G-1-P, 93 ± 1 %; F 1-P, 91 ± 6 %; ATP, 98 ± 6 %; PCr; 72 ± 2 %; F-1,6-P, 95 ± 2 % and glycogen, 68 ± 1 %.

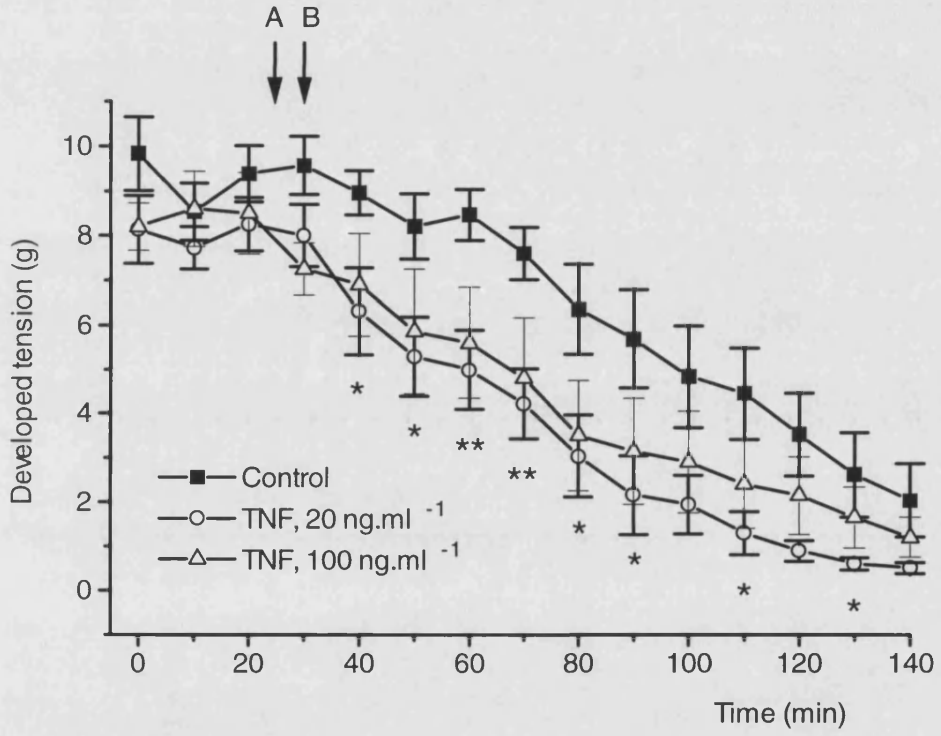
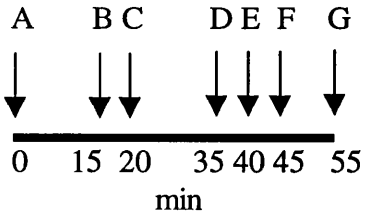


Figure 3.2. Effect of TNF, 20 ng.ml^{-1} (n = 6) and 100 ng.ml^{-1} (n = 4) on developed tension in the isolated heart perfused under recirculating conditions. TNF is added at A, recirculation begins at B. Control hearts (n = 6). * $P < 0.05$, ** $P < 0.01$, TNF, 20 ng.ml^{-1} vs. control.

3.2 Cardiac acidosis

As mentioned in the introduction, under certain acidotic conditions, TNF has the ability to insert itself into the plasma membrane, where there is potential for it to form ion permeable channels (Kagan *et al.*, 1992). For this reason it was decided to investigate whether the presence of TNF could alter the recovery of isolated hearts after an acidotic challenge following the protocol shown below:



A = initial perfusion, B = acidosis C = recovery with normal pH, D = TNF, 20 $ng.ml^{-1}$, or no treatment, E = application of acidosis, F = recovery with normal pH, G = end of perfusion.

Both degrees of acidosis, pH 5.5 and 6.1, produced very reproducible responses in developed tension, heart rate and CPP as shown by the experimental trace in figure 3.3. Recovery upon restoration of pH to 7.4 was assesed by developed tension, expressed as % of the developed tension before acidosis, and was measured 3 min and 7 min after acidosis. Prior inclusion of TNF to hearts 5 min before acidosis caused a slight decline in the recovery of these hearts from the acidotic challenge of pH 6.1 (figure 3.4) and pH 5.5. After a pH 5.5 challenge the recovery of control hearts at 3 min and 7 min was 59 ± 2 % and 85 ± 5 %, respectively vs. 56 ± 2 % and 74 ± 4 % with TNF (n = 4 for each).

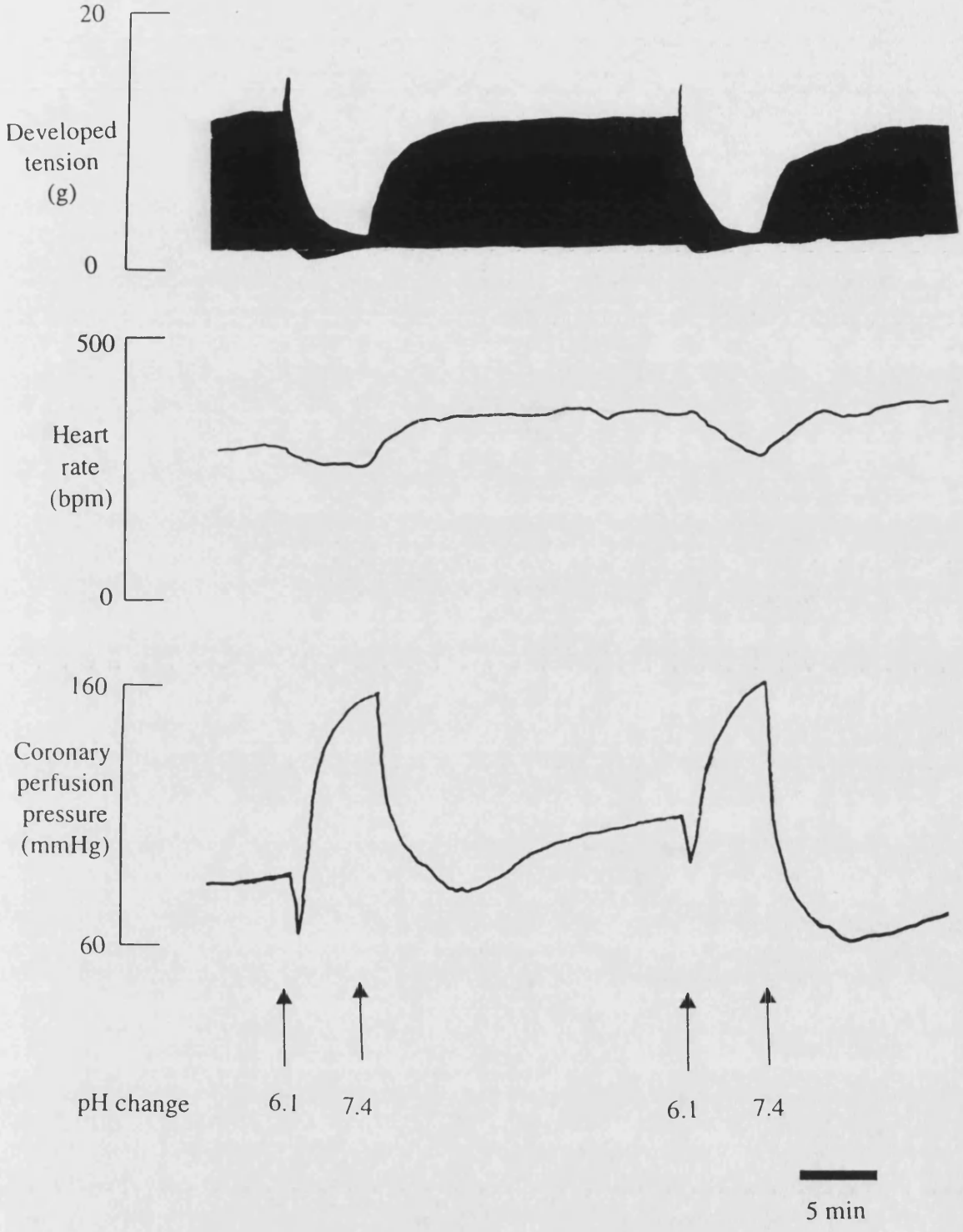


Figure 3.3.A typical experimental trace showing the changes in developed tension, coronary perfusion pressure and heart rate in the isolated perfused heart during and after two acidotic challenges.

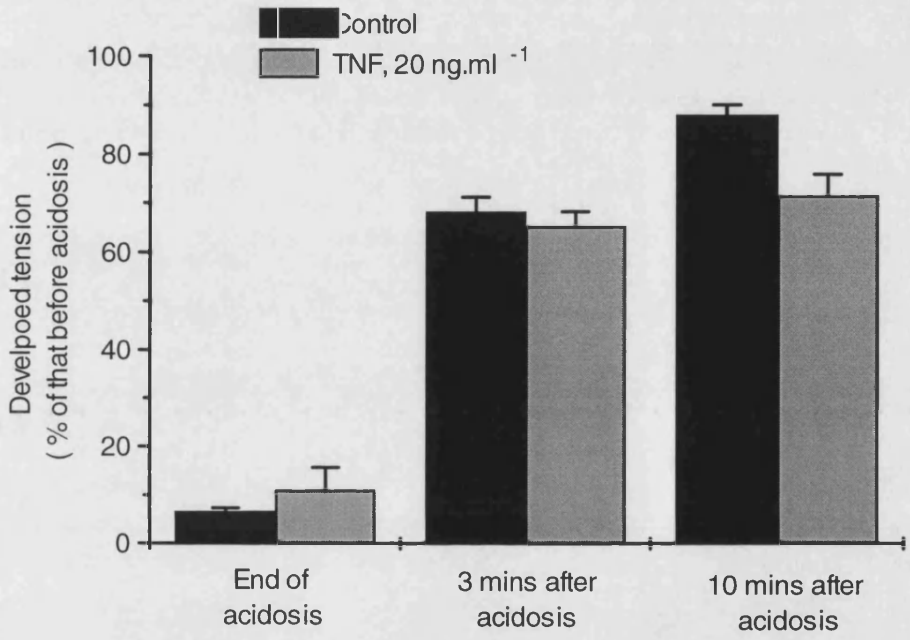


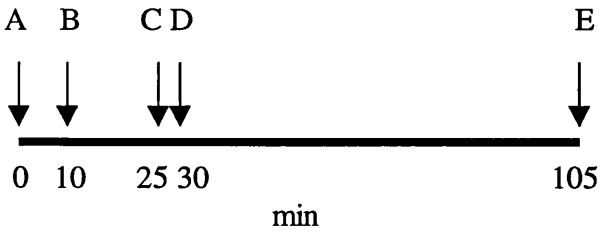
Figure 3.4. The above graph shows the recovery, in the presence and absence of TNF, of isolated hearts after a 5 min period of pH 6.1 acidotic challenge. (n = 4 for each point).

3.3 Low glucose recirculating heart

As discussed in the introduction, TNF has been shown to cause insulin resistance in whole animals (Lang *et al.*, 1992) as well as at the cellular level (Hotamisligil, *et al.*, 1994a; Begum & Ragolia, 1996). Therefore, hearts were perfused with low glucose (2 mM) containing Krebs buffer in order to observe the actions of insulin, where glucose uptake would be expected to show high insulin-dependency.

3.3.1 Insulin and TNF in the low glucose recirculating heart

Hearts were perfused according to the following protocol, then frozen to be assayed for glycogen:



A = initial perfusion, and addition of insulin, 10 $U.l^{-1}$, B = Low glucose, 2mM,
C = TNF, 20 $ng.ml^{-1}$, D = recirculation, E = end of perfusion

When perfused with Krebs buffer containing 2mM glucose and in the absence of TNF, developed tension decreased with time at a faster rate than in preliminary experiments using normal glucose (11.6 mM) concentration (figure 3.5). This faster decline in developed tension dictated a relatively shorter perfusion protocol. Figure 3.6 shows the actions of insulin and TNF alone and in combination on developed tension in this model.

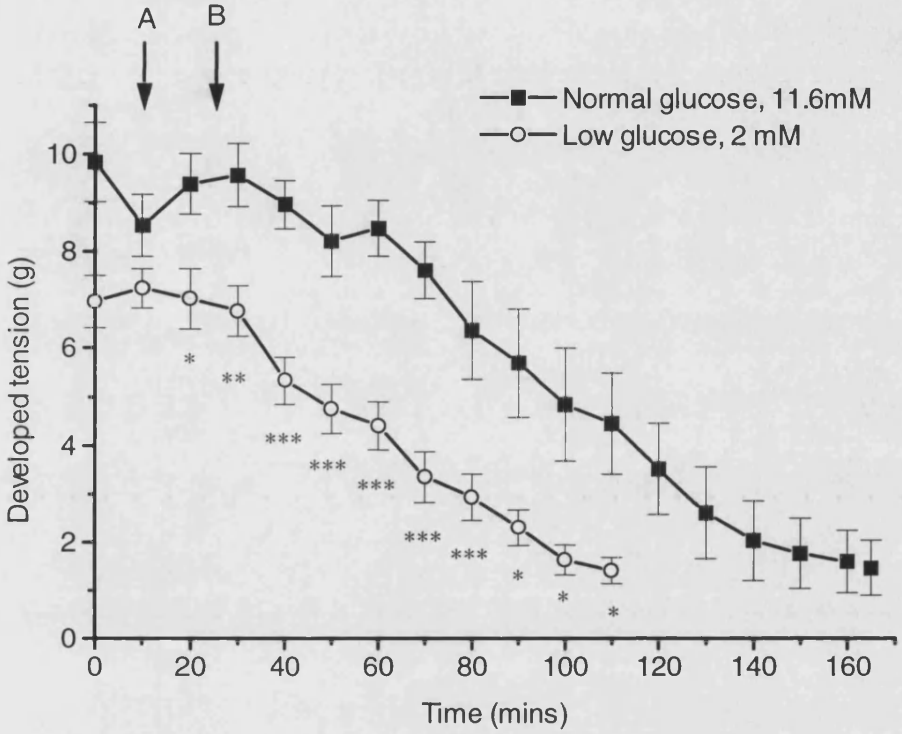


Figure 3.5. Effect of low glucose perfusion on developed tension in the isolated rat heart with time. Normal glucose, n = 6, low glucose, n = 12. Low glucose perfusion commenced at A, recirculation began at B. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, low glucose vs control

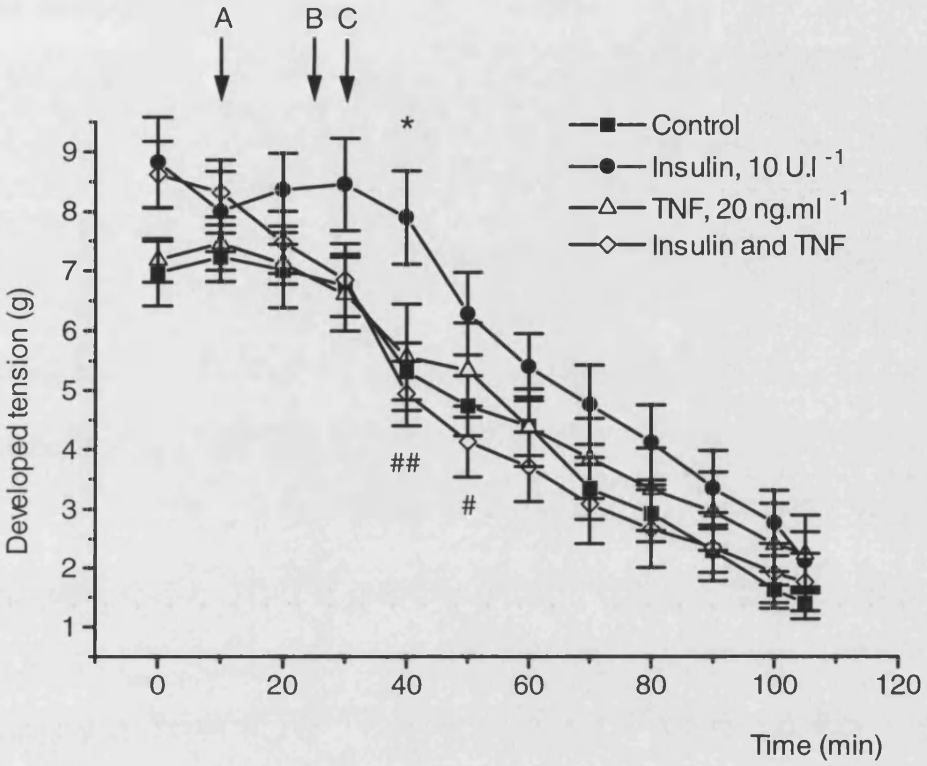


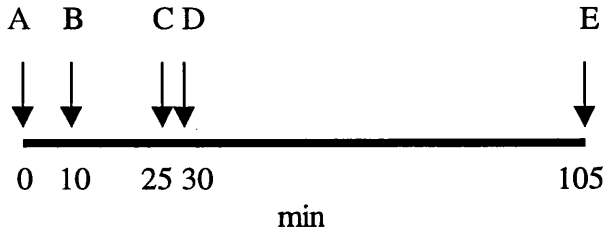
Figure 3.6. Actions of TNF (n = 10) and insulin (n = 11), alone and in combination (n = 12), on developed tension in the isolated rat heart perfused under low glucose conditions. Control hearts (n = 12). Low glucose (2mM) perfusion commenced at A. When present, TNF was added at B. Recirculation began at C. Insulin, was present throughout the protocol. * $P < 0.05$, insulin vs. control. # $P < 0.05$, ## $P < 0.01$, TNF and insulin vs. insulin alone.

At early time points after application of low glucose it can be seen that insulin slowed the decline in contractility, although this is only significant at one of the time points measured. After this developed tension in all groups studied decreased to a similar extent. Addition of TNF attenuated the early protection seen with insulin.

As expected perfusion of hearts, under low glucose conditions, with insulin caused a marked increase in glucose uptake, measured as decreasing glucose concentrations in the recirculating Krebs buffer (figure 3.7). TNF did not affect basal glucose uptake or the insulin stimulated glucose uptake (figure 3.7).

End-point glycogen levels in control hearts following low glucose perfusion were low (figure 3.8). Insulin caused a marked increase in end-point glycogen levels (figure 3.8). Interestingly TNF, when added 25 min after insulin, was able to partially prevent this insulin-stimulated glycogen synthesis. It can be seen from this graph that TNF did not affect end-point glycogen levels in the absence of insulin.

When insulin was added to hearts 20 min after TNF, very similar results to those described above were seen. Below is shown the diagrammatical representation of this protocol:



A = initial perfusion, and addition of insulin, 10 $U.l^{-1}$, B = Low glucose, 2mM,
C = TNF, 20 $ng.ml^{-1}$, D = recirculation, E = end of perfusion

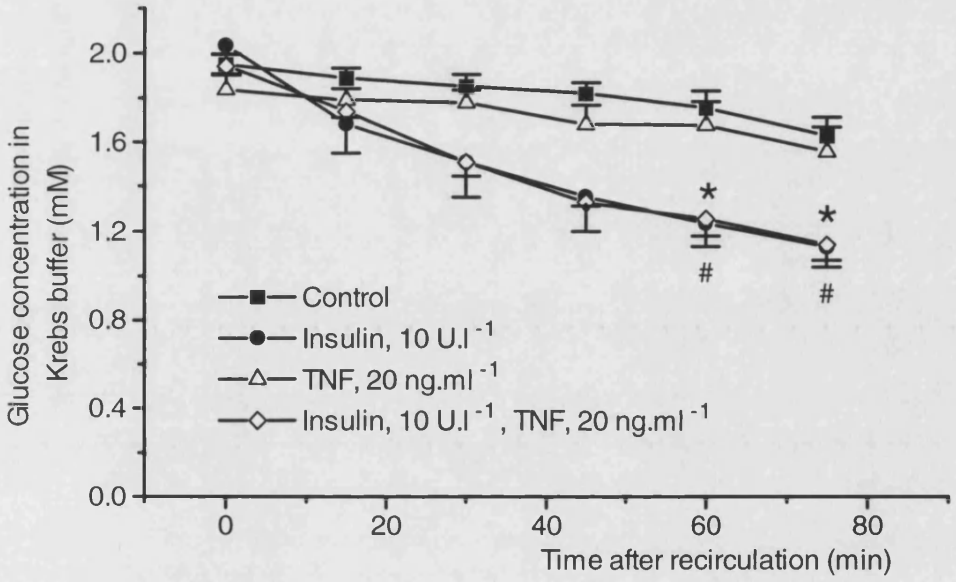


Figure 3.7. Glucose uptake from the recirculating Krebs buffer under low glucose (2mM) conditions, measured after recirculation, in control hearts (n = 6) and in TNF (n = 5), insulin (n = 5) and both TNF and insulin (n = 5) treated hearts. * $P < 0.05$; insulin vs. control. # $P < 0.05$; TNF vs. insulin and TNF.

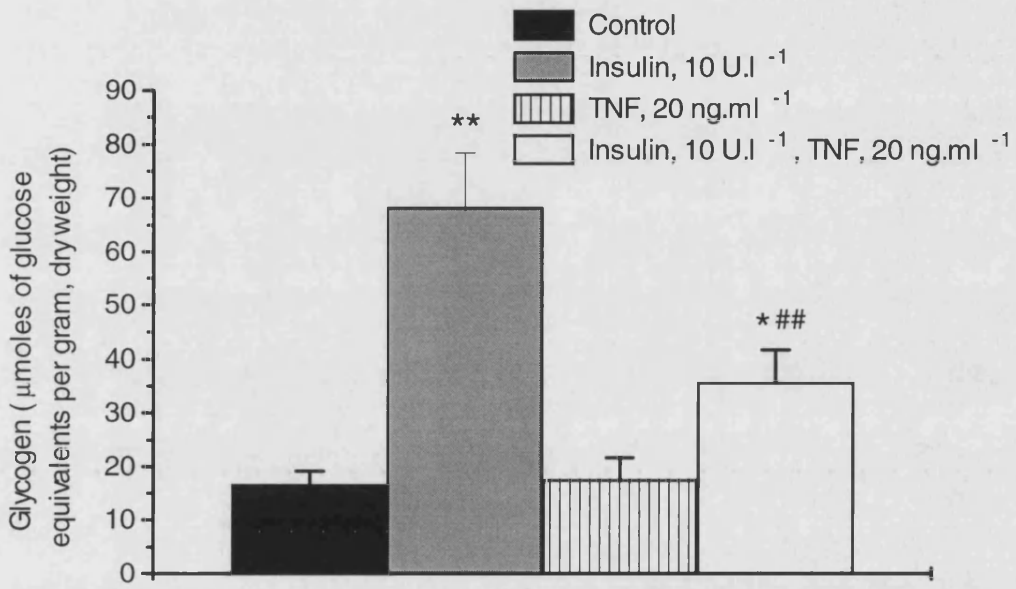
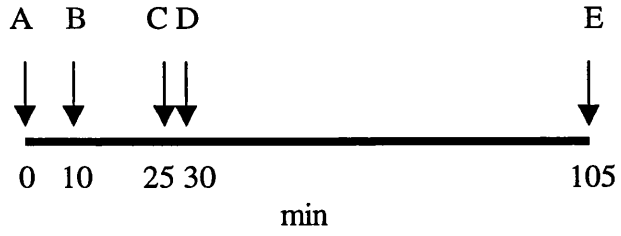


Figure 3.8. End-point glycogen levels in hearts perfused under low glucose conditions (n = 6) in the presence of insulin (n=6), TNF (n = 6) or both together (n = 6). * $P < 0.05$, ** $P < 0.01$ when compared with control. ## $P < 0.01$; TNF and insulin vs. insulin alone.

When added at this time point insulin again appeared to cause a slight protection from the early loss of function observed with a low glucose perfusion. This was not so in the presence of TNF (figure 3.9). However, it can be seen from the graph (figure 3.9), that at the point of recirculation, developed tension in hearts which were to have insulin added, was already slightly higher than in controls. At this time point, there had been no insulin treatment, and so developed tension in these hearts should be similar. Again, insulin caused a large increase in glucose uptake, which was not altered by TNF (figure 3.10). The insulin stimulated glycogen synthesis, was almost completely blocked by TNF (figure 3.11).

3.3.2 Ceramide and insulin in the low glucose recirculating rat heart

One of the potential signalling mechanisms utilised by TNF involves the sphingomyelinase pathway, where ceramide and sphingosine can act as second messengers. Ceramide has previously been shown to have the ability to disrupt insulin mediated responses (Begum & Ragolia, 1996), and so it was decided to try to mimic the actions, observed with TNF treatment, with a cell permeable form of ceramide, C_2-ceramide (C_2). The following protocol was used:



A = initial perfusion, and addition of insulin, 10 $U.l^{-1}$, B = Low glucose, 2mM,
C = C_2-ceramide, 1 μM, 5 μM or 10 μM, D = recirculation, E = end of perfusion

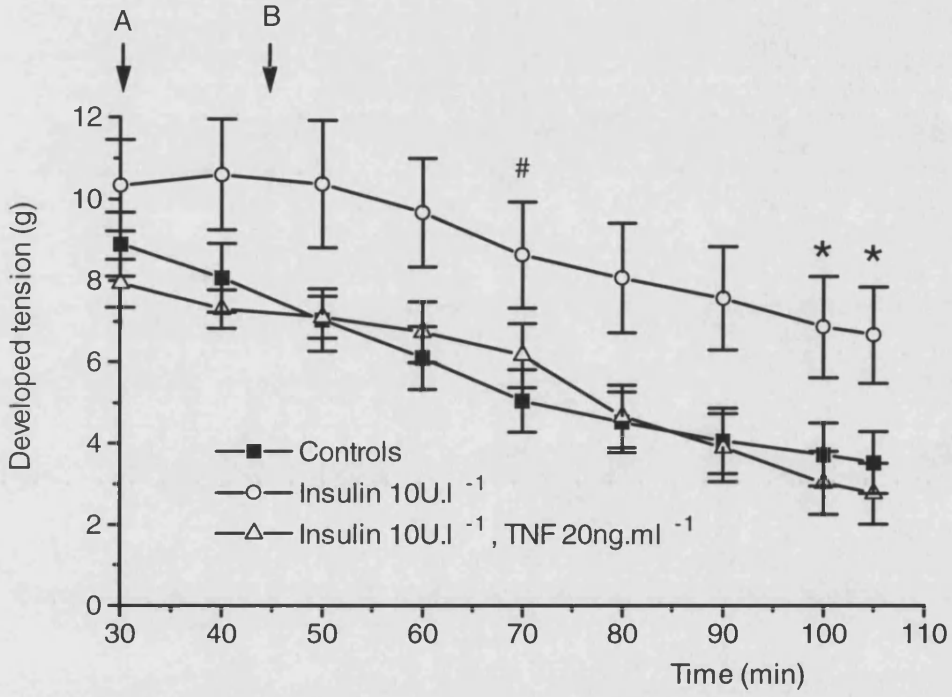


Figure 3.9. Actions of insulin and TNF on developed tension in the low glucose perfused heart where insulin was added after TNF. Recirculation began at A, and insulin was added at B. TNF, 20 ng.ml^{-1} was added 5 min prior to recirculation which began at A. * $P < 0.05$; insulin vs. control; # $P < 0.05$; TNF and insulin vs. insulin alone. (n = 6 for each).

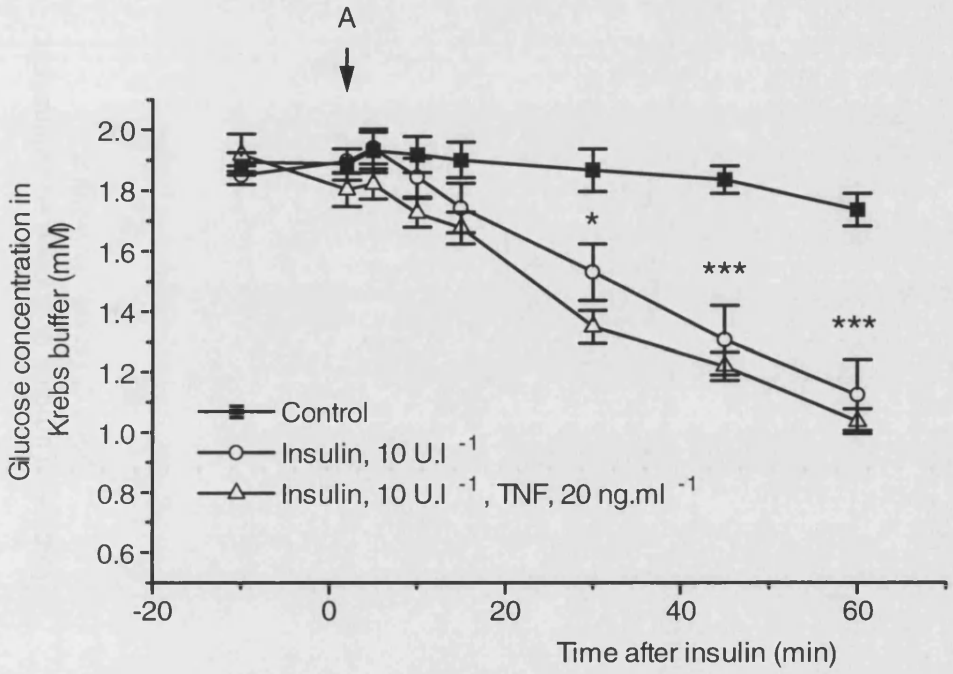


Figure 3.10. This graph shows the increase in glucose uptake seen upon insulin administration when insulin was after TNF. A shows the addition of insulin. This is shown both in the presence (n = 6) and absence (n = 6) of TNF. Control data, n = 6. * $P < 0.05$, *** $P < 0.001$; insulin vs. control.

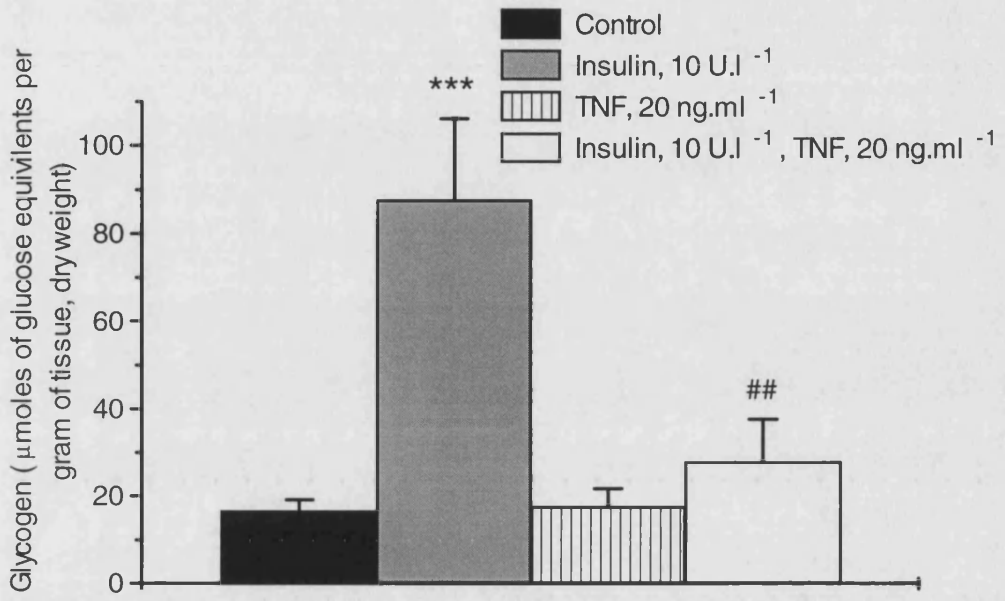
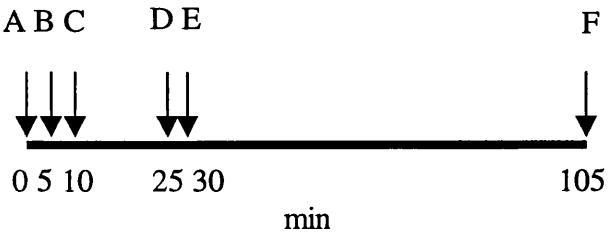


Figure 3.11. End-point glycogen levels in hearts perfused under low glucose conditions where insulin was added after TNF. *** $P < 0.001$; insulin vs. control. ## $P < 0.01$; TNF and insulin vs. insulin alone. (n = 8 - 12 for each group)

In the presence of insulin, C_2 caused a concentration-dependant depression in developed tension, whereby 1 μM C_2 did not adversely alter cardiac function, but both 5 μM and 10 μM C_2 had a marked negative inotropic effect (figure 3.12). As with TNF, none of the concentrations of C_2 used was able to alter glucose uptake from the Krebs buffer (figure 3.13). The actions of C_2 on insulin-stimulated glycogen synthesis were very interesting (figure 3.14). C_2, 1 μM, did not alter the end-point glycogen levels in the presence of insulin. However, both 5 μM and 10 μM of C_2 completely and significantly blocked the insulin-stimulated glycogen synthesis ($P < 0.001$). From these results a concentration of 5 μM C_2 was chosen for use of C_2 in subsequent experiments.

3.3.3 TNF, Ceramide and okadaic acid

To investigate the role of the ceramide-activated protein phosphatase (CAPP) in the blockade of glycogen synthesis by both TNF and C_2, the protein phosphatase inhibitor okadaic acid was used. At 1 nM okadaic acid shows a high degree of specificity towards the PP-2A subfamily of protein phosphatases, of which CAPP is one (Kolesnick, 1992). The following protocol was used:



i) A = initial perfusion, and addition of insulin, 10 $U.l^{-1}$ B = okadaic acid, 0.1nM or 1nM, C = Low glucose, 2mM, D = ceramide, 1μM, 5μM or 10μM, E = recirculation, F = end of perfusion, freeze clamp hearts.

ii) B = okadaic acid, 1nM, D = TNF 20 $ng.ml^{-1}$.

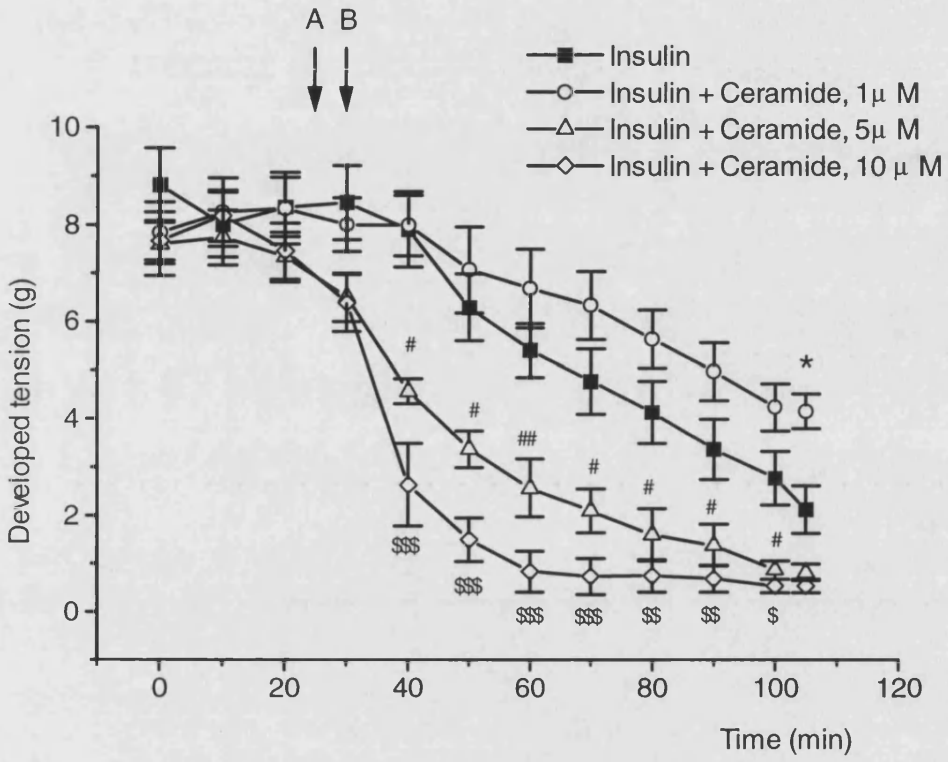


Figure 3.12. Effects of C_2-ceramide, 1 μM (n = 6), 5 μM (n = 6) and 10 μM (n = 6) on developed tension in the isolated rat heart. C_2-ceramide was added at A, recirculation commenced at B. * $P < 0.05$, 1 μM C_2-ceramide with insulin vs. insulin alone. # $P < 0.05$, 5 μM C_2-ceramide with insulin vs. insulin. $ $P < 0.05$, $$ $P < 0.01$, $$$ $P < 0.001$, 10 μM C_2-ceramide with insulin vs. insulin alone.

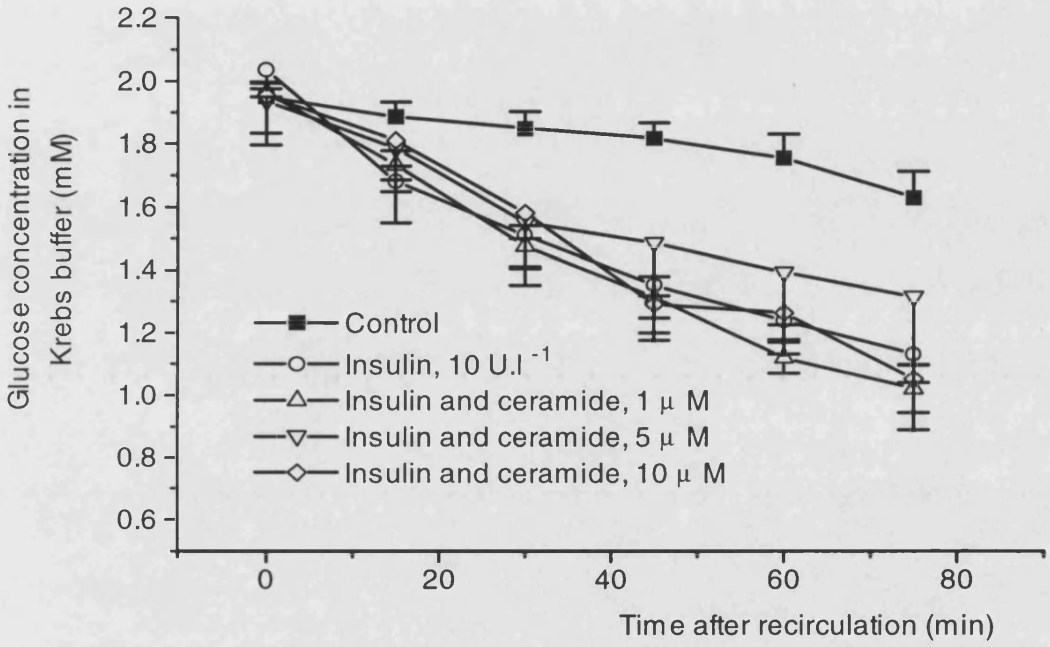


Figure 3.13. Effect of C_2-ceramide, 1 μM (n = 6), 5 μM (n = 6) and 10 μM (n=6), on insulin stimulated glucose uptake for the recirculating Krebs buffer. For significance levels of insulin effect, see figure 3.7.

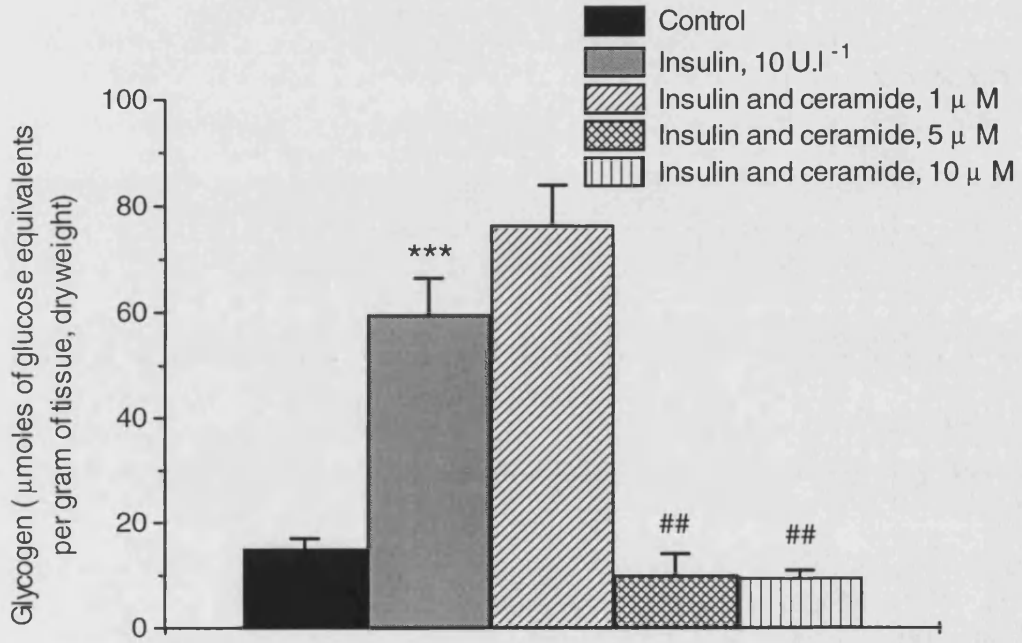
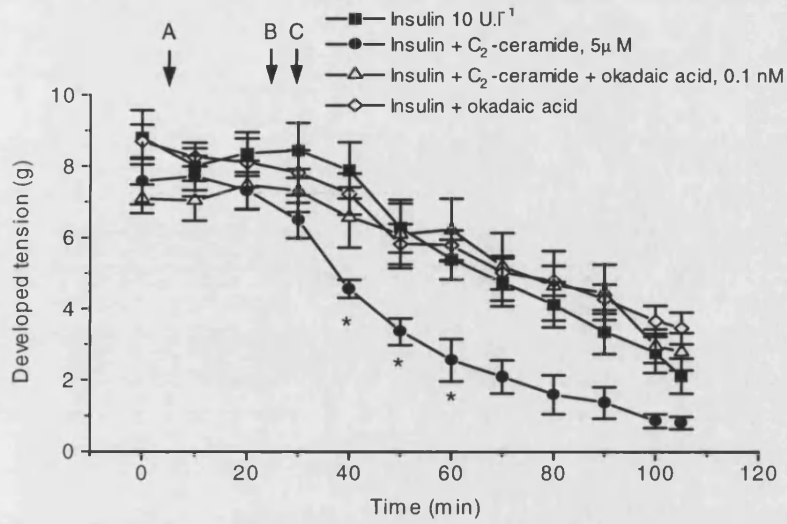


Figure 3.14. Effects of ceramide, 1 μM (n = 6), 5 μM (n = 6) and 10 μM (n = 6), on insulin stimulated glycogen synthesis in the isolated rat heart. *** $P < 0.001$, insulin vs. control. ## $P < 0.01$ ceramide and insulin vs. insulin.

In the absence of either ceramide or TNF, but in the presence of insulin, 1 nM okadaic acid increased cardiac contractility, this action was significant at 50 min and 60 min (figure 3.15). 0.1 nM okadaic acid did not alter cardiac contractility (figure 3.15). Figure 3.16 shows the actions of both C_2, 5 μM, and okadaic acid, 0.1 and 1nM, both alone and in combination on CPP in the isolated heart in the presence of insulin. It can be seen that 5 μM C_2 did not alter CPP. Both concentrations of okadaic acid used, either with or without C_2, showed a tendency to decrease CPP, but at none of these points was this action shown to be significant. Importantly, addition of okadaic acid at either concentration completely blocked the C_2-mediated disruption in insulin-stimulated glycogen synthesis, without significantly altering end-point glycogen levels in the absence of C_2 (figure 3.17).

However, when okadaic acid, 1 nM, was added prior to addition of TNF then it did not significantly alter the TNF-mediated disruption in insulin stimulated glycogen synthesis (figure 3.18).

a)



b)

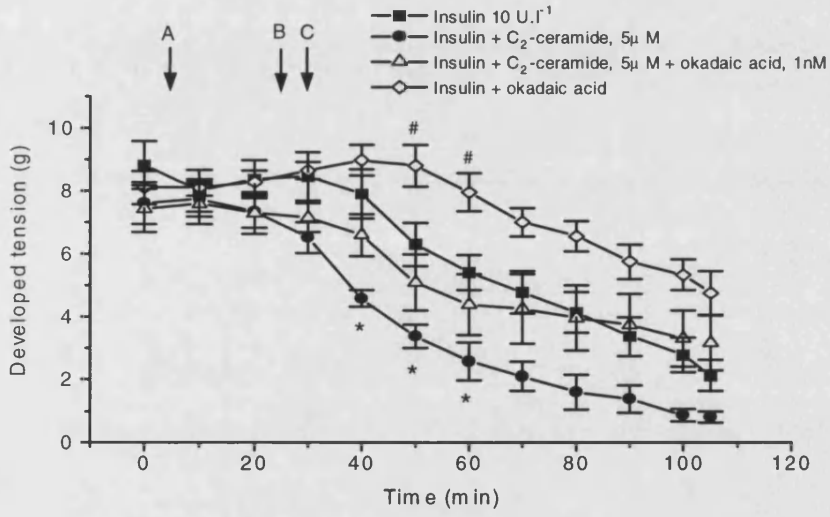
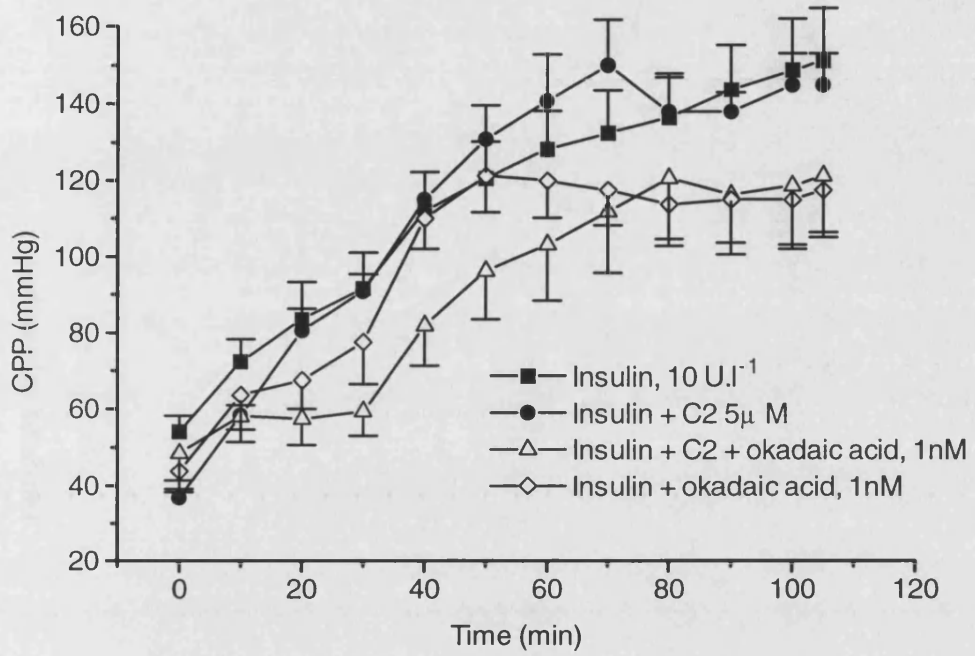


Figure 3.15. Effects of okadaic acid, 0.1 nM (a) and 1 nM (b), on changes in developed tension observed with C_2-ceramide, 5 μM, in the insulin treated low glucose perfusion model. Okadaic acid was added at A, C_2-ceramide was added at B and recirculation began at C. * $P < 0.05$, insulin and ceramide, with respect to insulin alone; # $P < 0.05$, insulin, okadaic acid and ceramide vs. insulin and okadaic acid. (n = 4 - 8 for ceramide and okadaic acid).



3.16. Actions of C_2-ceramide and okadaic acid, with time, on CPP in the rat isolated perfused heart. (n = 4 – 8).

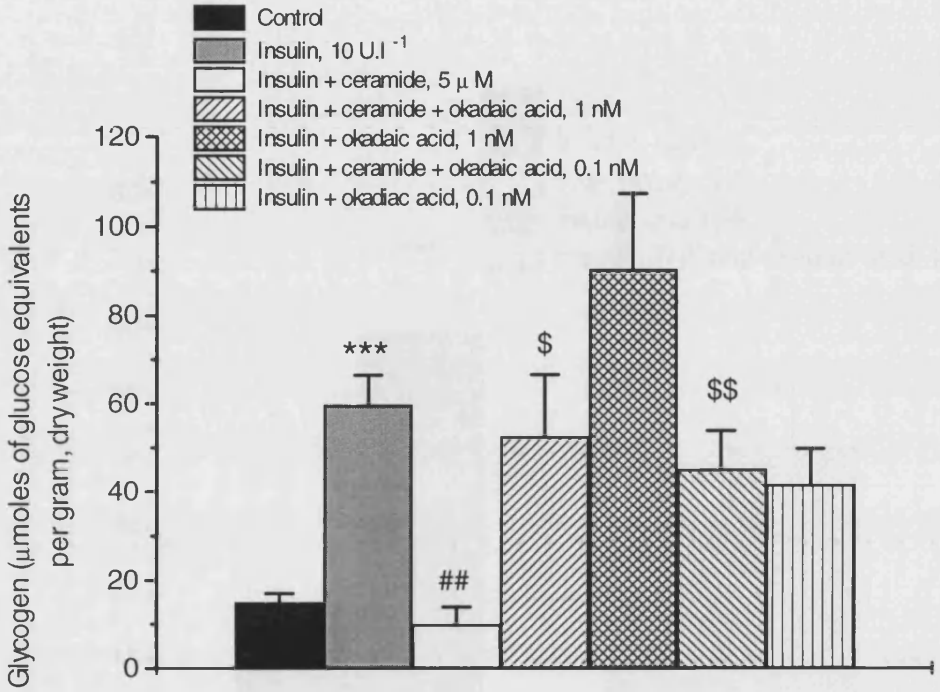


Figure 3.17. Effects of insulin and C_2-ceramide alone and in combination on end-point glycogen levels after low glucose perfusion. Also shown the effects of okadaic acid both in the presence and absence of C_2-ceramide on insulin stimulated glycogen synthesis. *** $P < 0.001$, insulin vs. control; ## $P < 0.01$, C_2-ceramide with insulin vs. insulin alone. \$ $P < 0.05$, \$\$ $P < 0.01$, okadaic acid, ceramide and insulin vs. ceramide and insulin. ($n = 4 - 8$ for ceramide and okadaic acid).

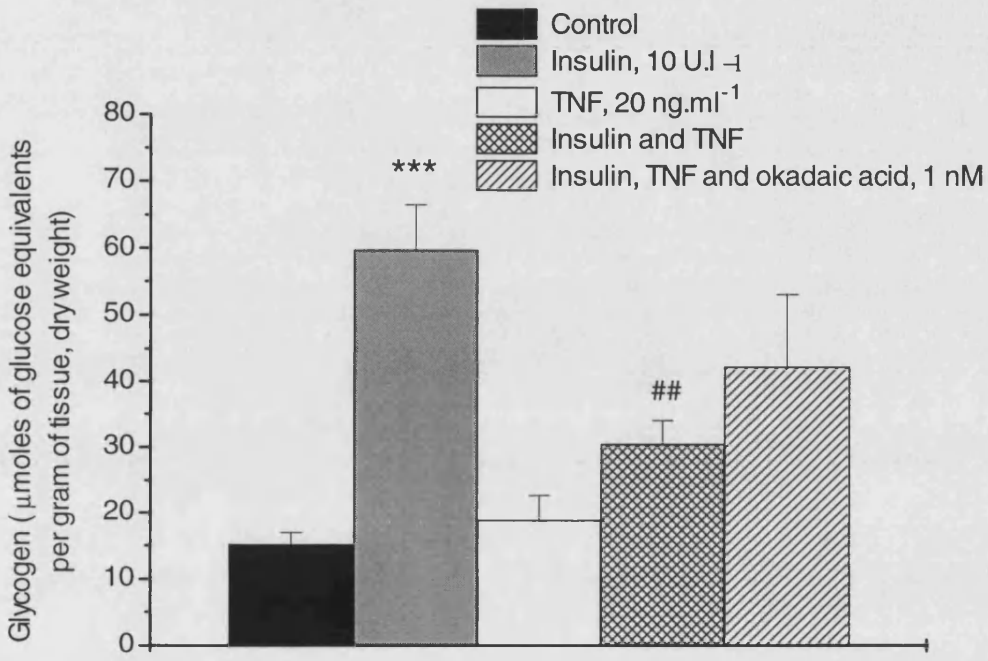
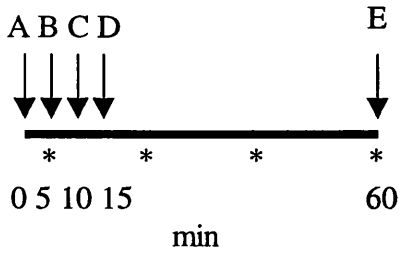


Figure 3.18. Effects of okadaic acid on the attenuation of insulin stimulated glycogen synthesis by TNF.*** $P < 0.001$, insulin vs. control; ## $P < 0.01$, TNF and insulin vs. insulin alone. (n = 8 - 12 for each group).

3.3.4 Glycogen depletion studies

Due to the observed actions of TNF on glycogen metabolism (see above), it was decided to see if TNF could alter glycogen breakdown in the absence of insulin. Under zero glucose conditions one would expect glycogen synthesis to be minimal, and glycogen breakdown would predominate. Hearts were perfused under zero glucose conditions in order to study the degradation of glycogen with and without TNF as shown below:



A = initial perfusion, B = zero glucose, C = TNF, 20 $ng.ml^{-1}$, D = recirculate,
E = end of perfusion.
* show the various points in the experiment where hearts were freeze clamped

Figure 3.19 shows glycogen depletion over the period of 60 min perfusion with no substrate. The remaining glycogen in control hearts was 73 %, 59 % and 20 % the initial content in hearts perfused with substrate-free medium for 15 min, 30 min and 60 min, respectively. The graph in figure 3.19 shows that TNF does not alter the rate of glycogen depletion under substrate-free perfusion.

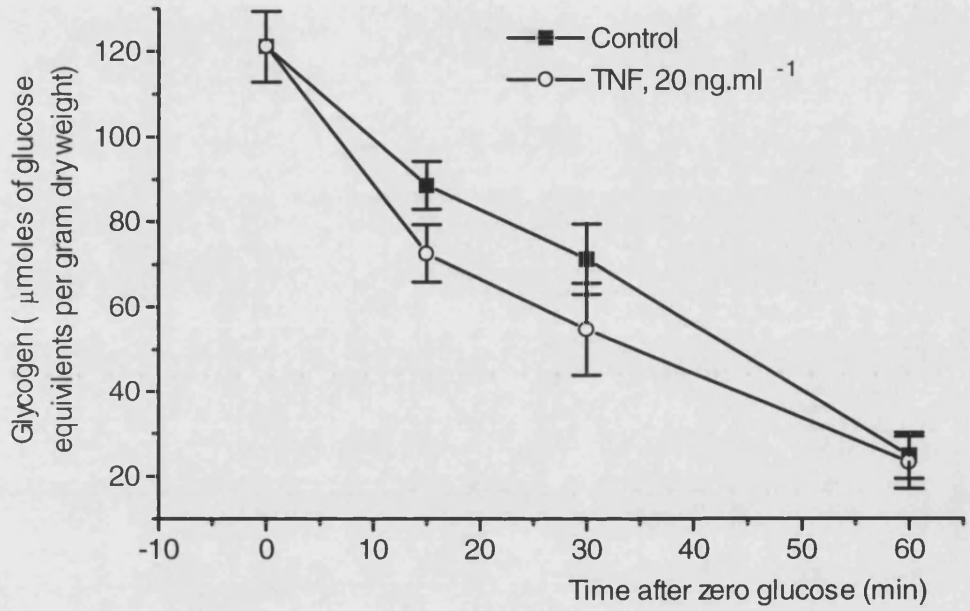
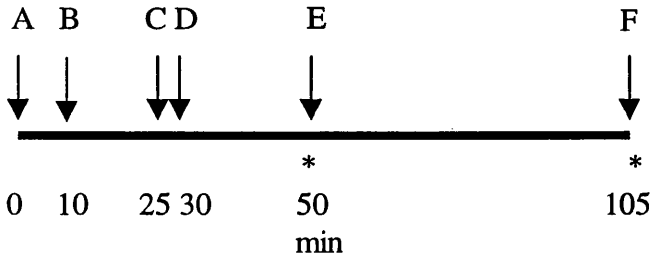


Figure 3.19. Glycogen depletion in hearts perfused with zero glucose in the presence and absence TNF. (n = 4 for each time point measured).

3.4 Low flow, low glucose ischaemic heart

The actions described above show that TNF can alter insulin action under conditions where substrate supply would be limiting. For this reason it was decided to investigate the actions of TNF under conditions of further substrate deprivation. Here global low flow ischaemia (2 $ml.min^{-1}$) under low glucose (2 mM) conditions was used according to the following protocol:



A = initial perfusion, B = Low glucose, 2mM, C = TNF, 20 $ng.ml^{-1}$, D = recirculation, E = Low flow ischaemia, 2 $ml.min^{-1}$, F = end of perfusion.

* show the two points in the experiment where hearts were freeze clamped.

As well as end-point glycogen levels, end-point ATP and PCr levels were also measured.

Another reason for using an ischaemic model to investigate the actions of TNF stems from studies suggesting that TNF can play a detrimental role during ischaemia (Colletti *et al.*, 1990; Meldrum *et al.*, 1998).

3.4.1 Actions of TNF during global low flow ischaemia under low glucose conditions

In hearts subjected to global low flow, 2 $ml.min^{-1}$, developed tension dropped as soon as ischaemia was initiated, and at no point did the developed tension recover (figure 3.20). Inclusion of insulin, 10 $U.l^{-1}$, from the start of the experiment slightly slowed the decline in developed tension, however, this was only significant at the first time point after the start of ischaemia, 10 min). Addition of TNF 5 min before recirculation showed unexpected results. The rapid decrease in developed tension observed upon recirculation was not as great as in control hearts, and developed tension remained elevated throughout the ischaemic period. In hearts perfused with both TNF and insulin developed tension was again significantly elevated during low flow ischaemia, although the response was not significantly different from either TNF or insulin alone.

As expected, at the onset of global low flow ischaemia, there was a rapid fall in CPP, which was not significantly different between each group (decrease in CPP after initiation of ischamia for control hearts, insulin treated, TNF treated and insulin with TNF treated hearts: 43 ± 3 mmHg, 61 ± 14 mmHg, 49 ± 9 mmHg and 53 ± 12 mmHg respectively, P = NS between any groups, n = 6 for each group.

After the onset of ischaemia, a contracture developed, manifested as an increase in baseline tension (figure 3.21). Neither TNF nor insulin, alone or in combination, significantly altered the observed contracture, but several trends were apparent. Insulin appeared to slightly slow the development of contracture (figure 3.21). TNF, tended to

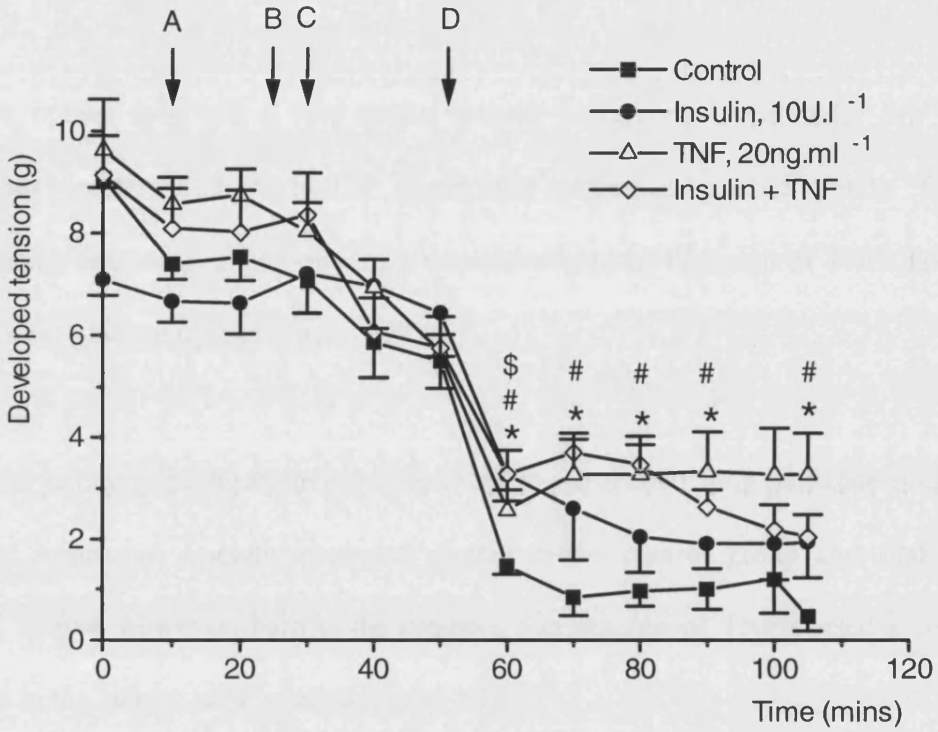


Figure 3.20. Effects of TNF and insulin, alone or in combination on developed tension in the isolated perfused rat heart under low glucose conditions, and subjected to global low flow ischaemia. Low glucose perfusion began at A, TNF was added at B and recirculation commenced at C. Low flow ischaemia (2 $ml.min^{-1}$) was initiated at D. Insulin was present from the start of the perfusion protocol. * $P < 0.05$ TNF vs. control. # $P < 0.05$ insulin and TNF vs. control. $ $P < 0.05$ insulin vs. control n = 6 for each group.

cause a small decrease in the severity of contracture, although this was not shown to be significant (figure 3.21).

3.4.2 Glucose uptake and metabolic changes

Glucose uptake followed a very similar pattern to that observed under low glucose conditions (section 3.3.1). Insulin significantly increased glucose uptake from the recirculating perfusate, an action which was not altered by inclusion of TNF. TNF alone did not alter glucose uptake (figure 3.22).

Very low lactate concentrations were observed in the recirculating perfusate prior to the onset of ischaemia. Lactate increased slightly in the control group and TNF treated groups. Insulin, however, both in the presence and absence of TNF caused a significant increase in the lactate accumulation (figure 3.23).

Neither ATP or PCr changed significantly during the ischaemic period in control hearts. However, with TNF and insulin, both alone and in combination, there was a significant decline in ATP and PCr over the ischaemic period (table 3.1). Both ATP and PCr end-point levels were very low in hearts treated with both TNF and insulin, these values were significantly lower than either the control group or the TNF treated group.

Ischaemia caused a decline in glycogen content of the hearts, but there was no difference between any of the groups (table 3.1). Interestingly glycogen levels observed in these

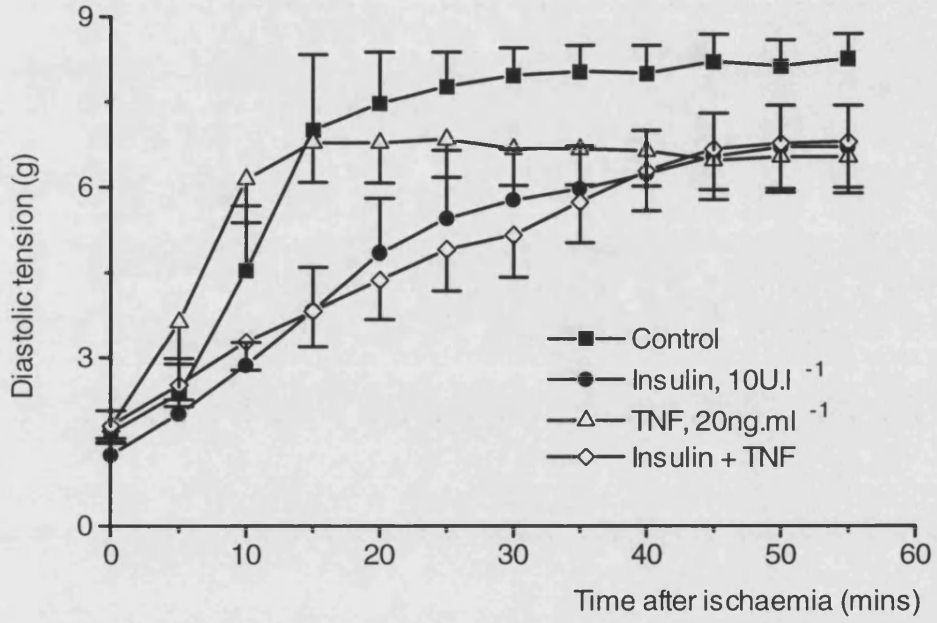


Figure 3.21. Effects of TNF and insulin, alone and in combination on the diastolic tension following the onset of global low flow (2 ml.min^{-1}), low glucose (2mM) ischaemia. n = 6 for each group.

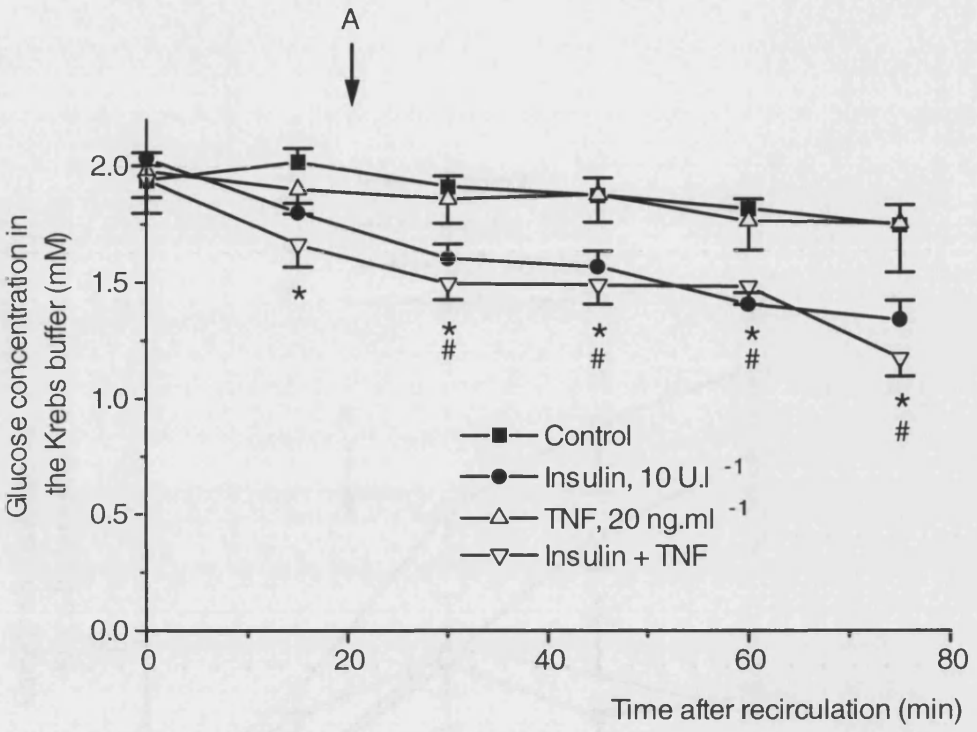


Figure 3.22. Glucose uptake from the recirculating Krebs buffer before and during global low flow ischaemia in the presence of TNF, insulin or both together. Low flow ischaemia was initiated at A. * $P < 0.05$ insulin vs. control. # $P < 0.05$ TNF and insulin vs. TNF alone ($n = 6$ for each group).

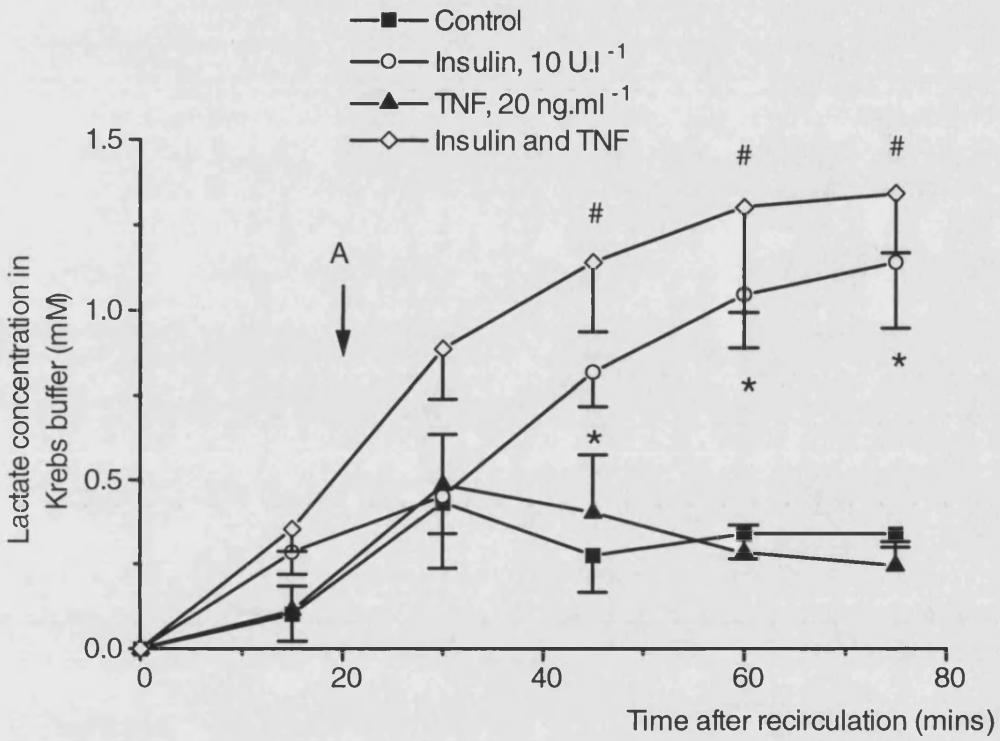


Figure 3.23. Effects of insulin and TNF alone and in combination on lactate accumulation in the recirculating perfusate. Recirculation began at time 0, low flow ischaemia (2 ml.min^{-1}) was initiated at A. * $P < 0.05$ insulin vs. control. # $P < 0.05$ TNF and insulin vs. TNF alone. n = 6 fro each group.

	ATP, μmoles per gram of tissue, dry weight.		PCr, μmoles per gram of tissue, dry weight.		Glycogen, μmoles of glucose equivalents per gram of tissue, dry weight.	
	Pre-ischaemia	Post-ischaemia	Pre-ischaemia	Post-ischaemia	Pre-ischaemia	Post-ischaemia
Control	21 ± 3	12 ± 3	14 ± 4	18 ± 2	93 ± 19	41 ±10 *
Insulin, 10 $U.l^{-1}$	35 ± 12	12 ± 2 *	21 ± 7	9 ± 1 *	123 ± 21	38 ± 9 *
TNF, 20 $ng.ml^{-1}$	46 ± 6	16 ± 1 *	38 ± 15	9 ± 1 *	105 ± 17	50 ± 9 *
Insulin + TNF	39 ± 7	9 ± 1 *#	34 ± 11	4 ± 1 *$#	90 ± 19	51 ± 7 *

Table 3.1. The above table shows a comparison between pre- and post- ischaemic ATP, PCr and glycogen levels in low glucose hearts in the presence of insulin, TNF or both together. * $P < 0.05$ post-ischaemic vs. pre-ischaemic. $ $P < 0.05$ insulin and TNF vs. TNF alone. # $P < 0.05$ insulin and TNF vs. control. ($n = 4$ for pre-ischaemic groups, $n = 6$ for post ischaemic groups).

studies were higher in control and TNF treated hearts which had undergone an ischaemic episode than in hearts which followed the low glucose perfusion (table 3.1 vs. figure 3.8). However in the presence of insulin, end-point glycogen levels were lower after global low flow ischaemia ($P < 0.05$).

3.5 Actions of TNF in the isolated perfused heart, under constant flow conditions

As mentioned in the introduction, TNF has the ability to cause a depression in cardiac function, an action for which various mechanisms have been proposed (Finkel *et al.*, 1992, Shultz *et al.*, 1995, Goldhaber *et al.*, 1996, Oral *et al.*, 1997). So the actions of TNF on cardiac function were investigated in hearts perfused with Krebs buffer containing 11.6 mM glucose, where the energy substrate, glucose, is in plentiful supply. The protocol followed is shown below:

A = initial perfusion, B = antagonist or inhibitor, C = TNF, 20 ng.ml^{-1},
D = recirculation, E = end of perfusion (however with all inhibitors except nitro-L-arginine, perfusion time was cut to 90 min)
* show where Starling curves were performed

3.5.1 Effects of TNF on cardiac contractility

Results from preliminary experiments, figure 3.2, indicated that in my hands, TNF (20 ng.ml^{-1}) caused an early and sustained depression in contractility, measured by developed tension via a hook in the apex of the heart. It was decided that left ventricular developed

pressure (LVDP), measured using a fluid filled intraventricular balloon, was a superior indicator of cardiac contractility, than developed tension measured with a hook. Results presented in the following section have all been obtained using this model.

It can be seen from the experimental traces shown in figure 3.24, as well as the graph shown in figure 3.25, that as in preliminary experiments, cardiac contractility decreased in control hearts throughout the 160 min perfusion protocol. It should be noted that recirculation had no immediate significant effect on LVDP (LVDP before recirculation vs. after recirculation: 127 ± 3 mmHg vs. 120 ± 5 mmHg, P = NS). Importantly, as in preliminary experiments, addition of TNF 20 $ng.ml^{-1}$ to the perfusate resulted in a depression in cardiac function, which persisted throughout the experimental protocol (figure 3.24 and 3.25). Rate of change of both systolic contraction and diastolic relaxation were also both depressed, shown in figure 3.26, although the depression in both differential parameters of LVDP took slightly longer to reach statistical significance (TNF treated vs. control) than the direct measure of LVDP.

TNF had no immediate or late, chronotropic effect (heart rate before TNF vs. after TNF: 269 ± 11 beats per minute (bpm) vs. 279 ± 8 bpm, P = NS, n = 10 for each. Heart rate after 160 min: control vs. TNF; 270 ± 20 bpm vs. 280 ± 15 bpm. P = NS, n = 10 and n = 8 respectively). For changes in CPP with TNF in this model see section 3.6.

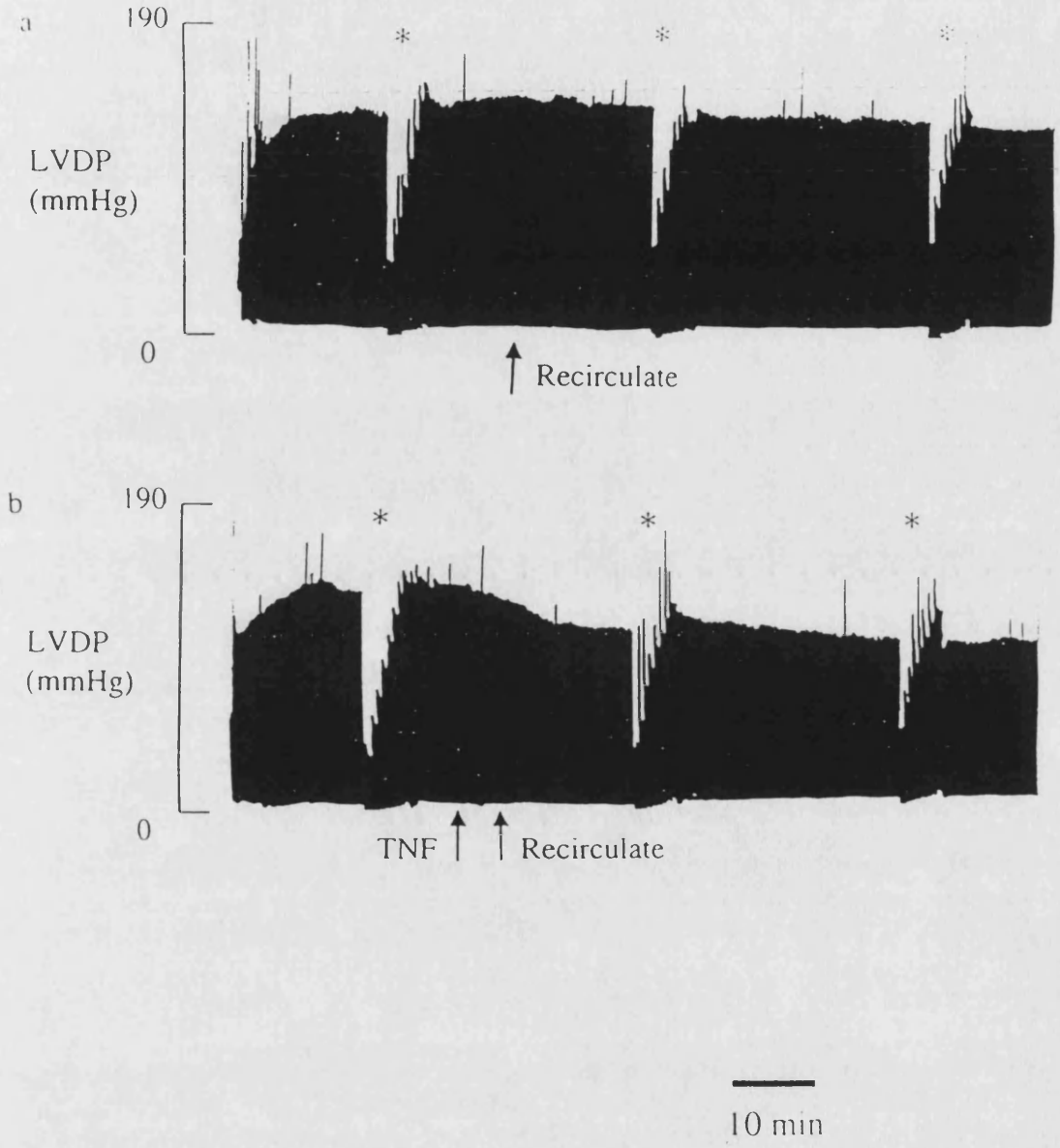


Figure 3.24. Typical experimental traces showing LVDP changes over a period of 90 min for both control hearts (n = 13), and TNF, 20 ng.ml^{-1} (n = 10). Starling curves are indicated by *.

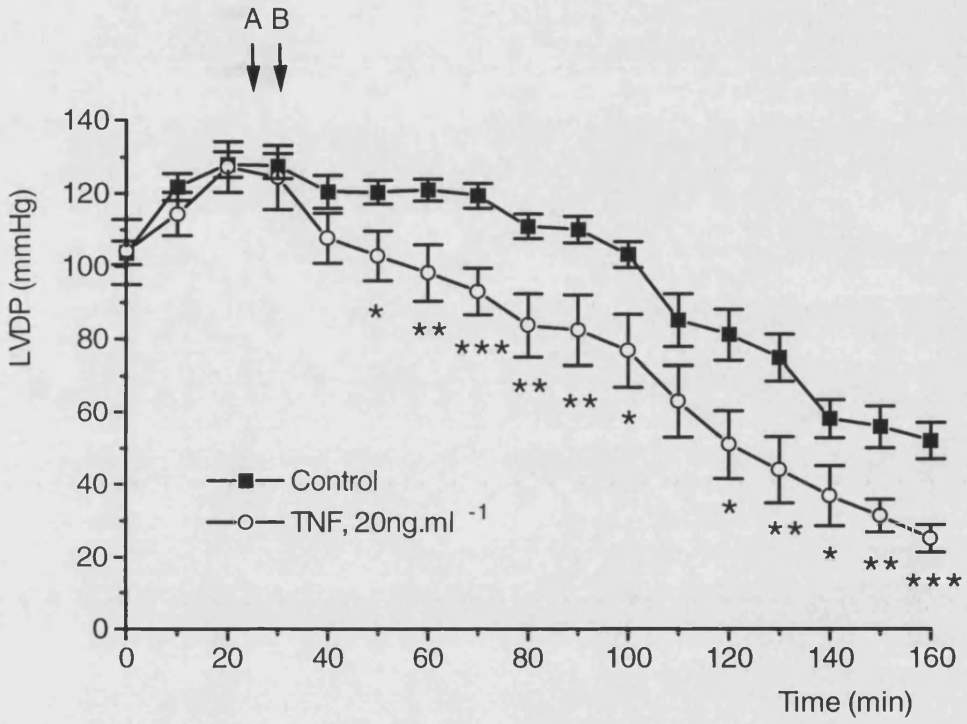
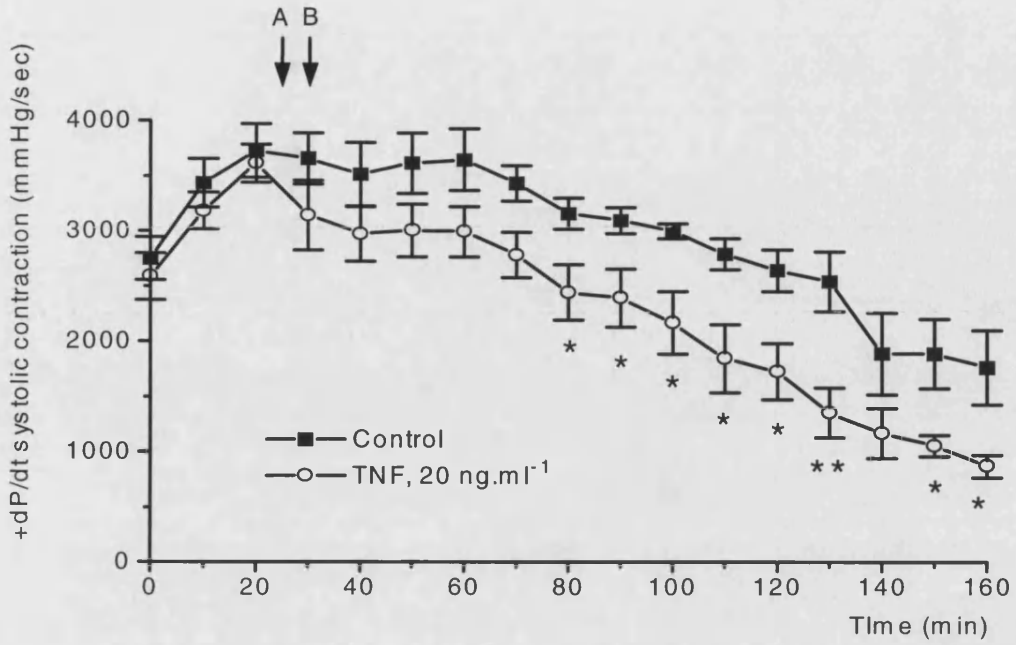


Figure 3.25. Effects of TNF on the decline in LVDP with time in the isolated rat heart under constant flow (10 ml.min^{-1}) conditions. TNF was added at A, recirculation commenced at B. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; TNF vs. control. n = 13 for control hearts and n = 10 for TNF treated hearts.

a)



b)

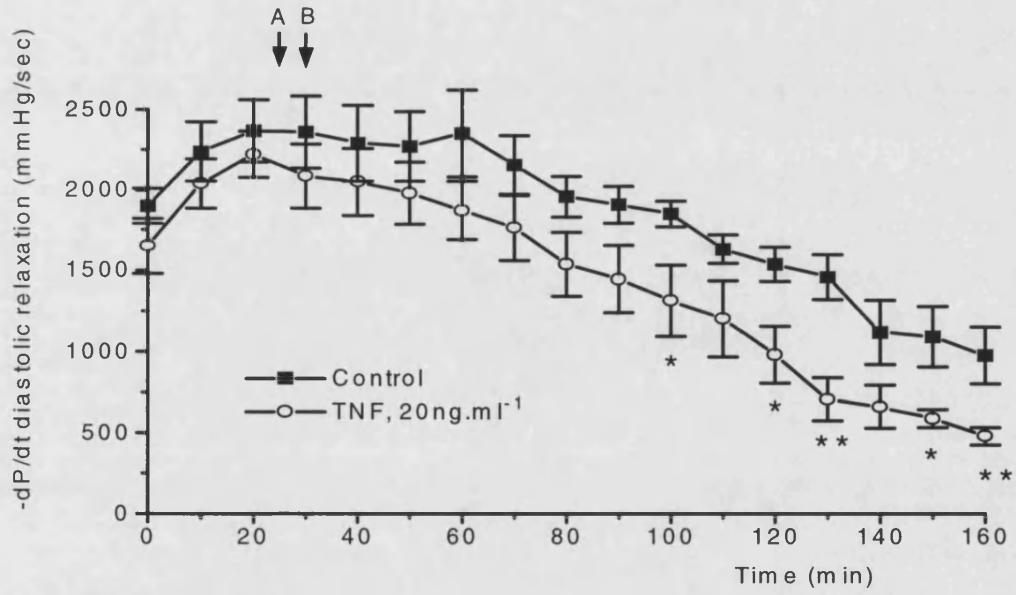


Figure 3.26. The above graphs show the rate of change of systolic contraction, a) and the rate of change of diastolic relaxation, b) in control hearts (n = 7) and hearts treated with TNF (n = 9). TNF is given at A and recirculation began at B. * $P < 0.05$, ** $P < 0.01$; TNF vs. control.

3.5.2 Starling curves

As mentioned earlier, one of the major advantages of using a balloon to measure left ventricular contractility is that Starling curves can be performed in perfused hearts. A typical experimental trace of a Starling curve is shown in figure 3.27. A profile of Starling curves, comparing TNF treated hearts with control hearts, is shown in figure 3.28. It can be seen that prior to TNF addition, the Starling curves performed at 15 min were very reproducible when comparing both groups of hearts. Thereafter, however, TNF treatment lead to a progressive decline in the hearts ability to respond to an increase in left ventricular diastolic volume. This reached significance during the fifth and sixth Starling curves, performed at 135 min and 165 min. Again, as with the differential parameters of LVDP, this was at a later time point than the decline in contractile performance when measured directly using LVDP.

3.5.3 Perfusion with high glucose concentration

Under normal glucose conditions (11.6 mM), TNF caused an early depression in function, which was apparent after 25 min which was sustained throughout the experimental procedure. However, in previous studies with a low glucose (2 mM) perfusion model no such depression was observed (see above). Therefore, it was decided to investigate whether this depression in function was dependant on the glucose concentration and whether the depression in function could be potentiated with increased glucose concentrations. Indeed glucose has been shown to facilitate NO production (Sobrevia *et*

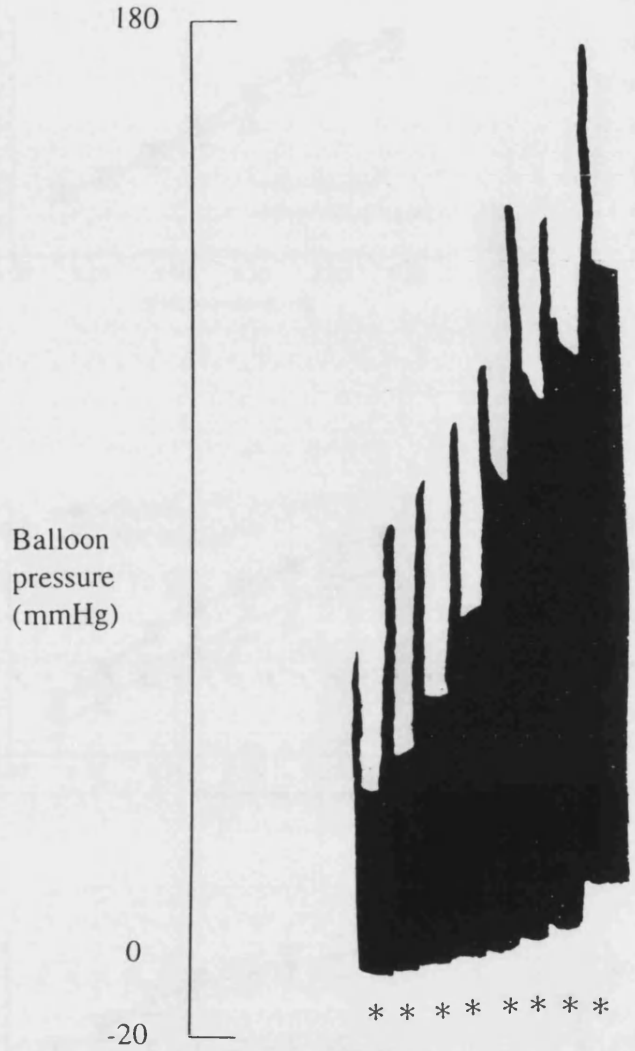


Figure 3.27. The above experimental trace shows changes in balloon pressure during a typical Starling curve performed in an isolated perfused heart. * shows a volume increase in the balloon of 0.03 ml.

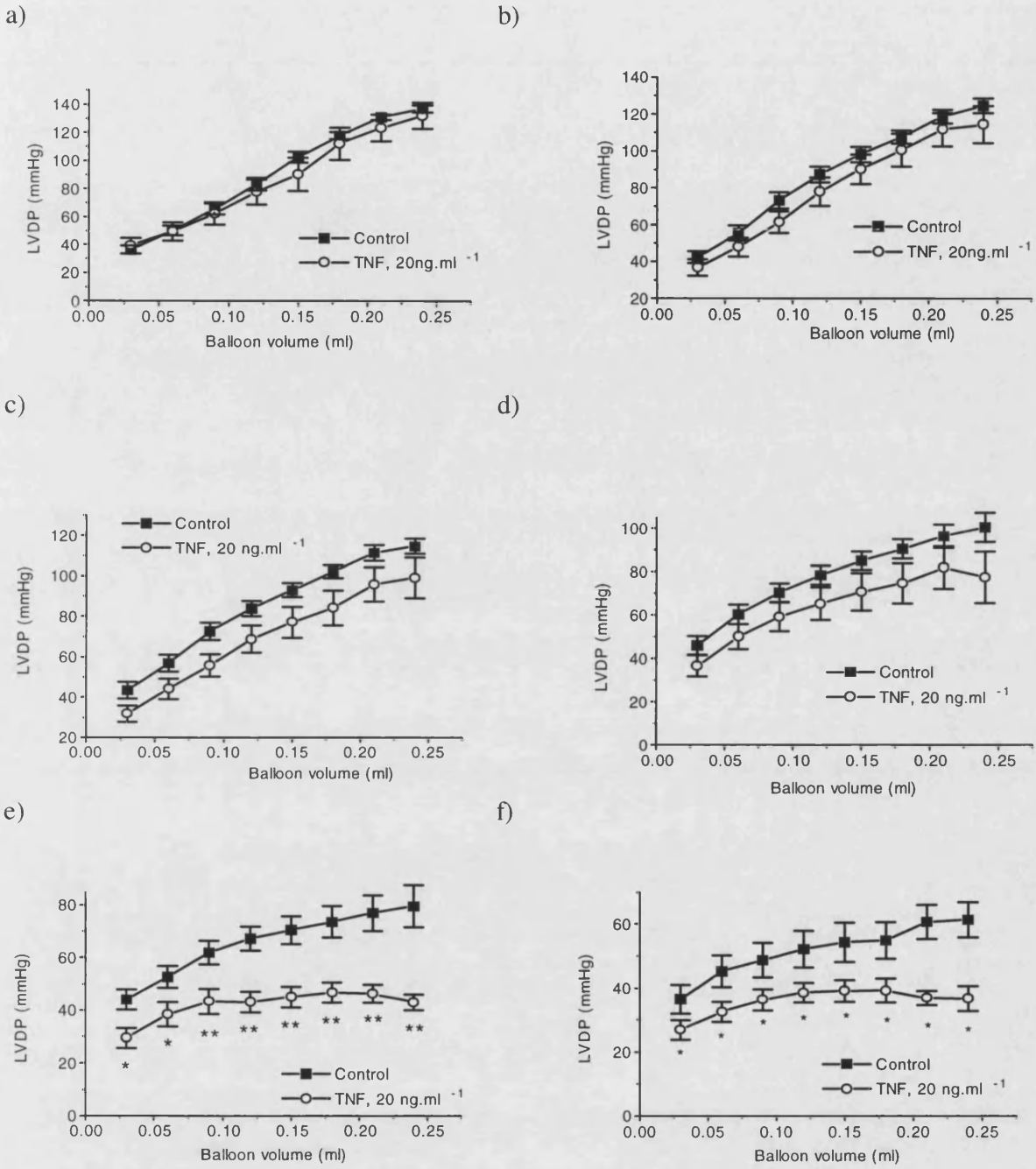
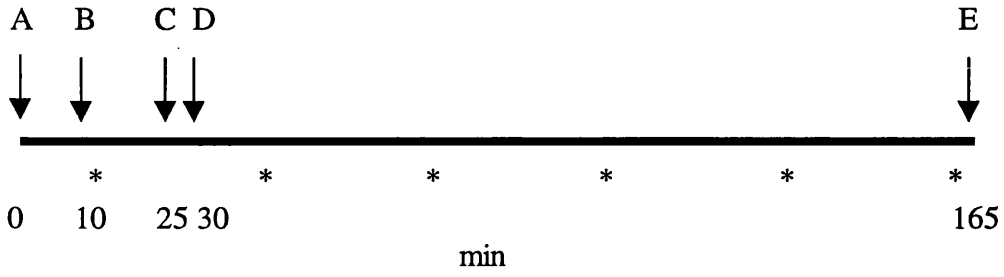


Figure 3.28. Profile of Starling curves performed in the isolated perfused heart at 15 min (a), 45 min (b), 75 min (c), 105 min (d), 135 min (e) and 165 min (f), in the presence and absence of TNF. * $P < 0.05$, ** $P < 0.01$; TNF vs. control (n = 8 - 13 for each point shown).

al., 1996; Costentino *et al.*, 1997), and form relatively stable NO-donating compounds, which would prolong the short half-life of NO (Moro *et al.*, 1995). The following protocol was used:



A = initial perfusion, B = high glucose perfusion, 25 mM, C = TNF, 20 ng.ml^{-1}, D = recirculation, E = end of perfusion, hearts freeze clamped.

* show where Starling curves were performed

Perfusion of isolated hearts with Krebs buffer containing a high glucose concentration, 25 mM, did not alter the tendency for a decline in cardiac function throughout the 160 min perfusion protocol (figure 3.29). The slow decline in cardiac function seen with recirculation was similar in all respects to that seen under a normal glucose conditions. The cardiac depression seen with TNF under normal glucose conditions was also apparent with a high glucose perfusion (figure 3.29), although this only reached significance ($P < 0.05$) at one of the time points measured. This was probably due to a lower number of experiments conducted with high glucose. However, it can be seen from this figure, that the profile of depression followed a very similar magnitude and time course to that seen with normal glucose. From these experiments it is clear that high glucose did not potentiate the cardiac depressant actions of TNF.

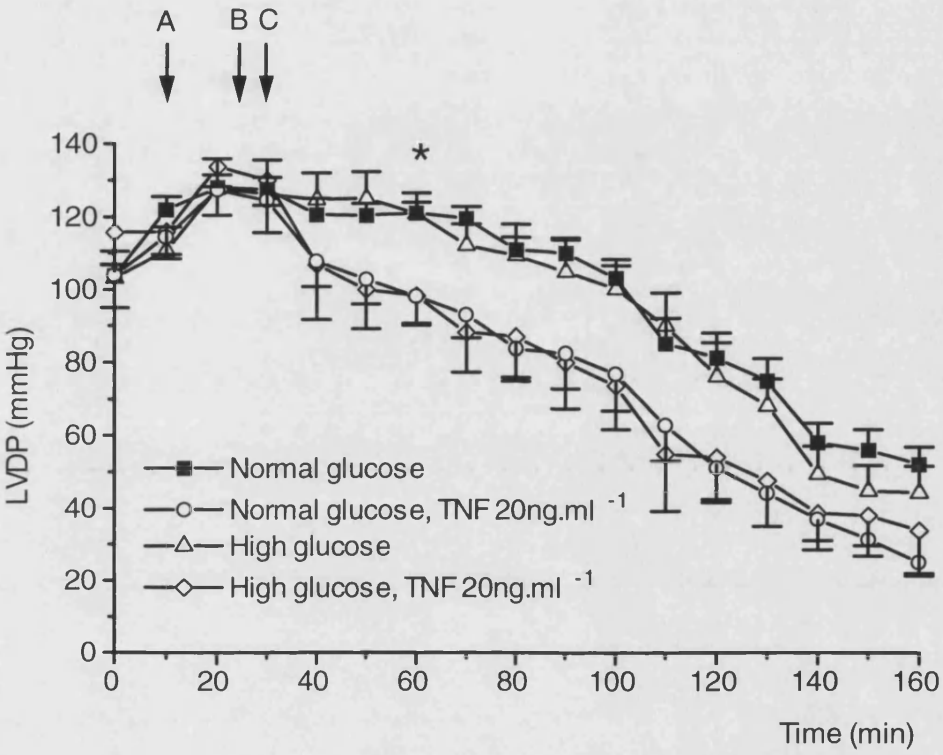
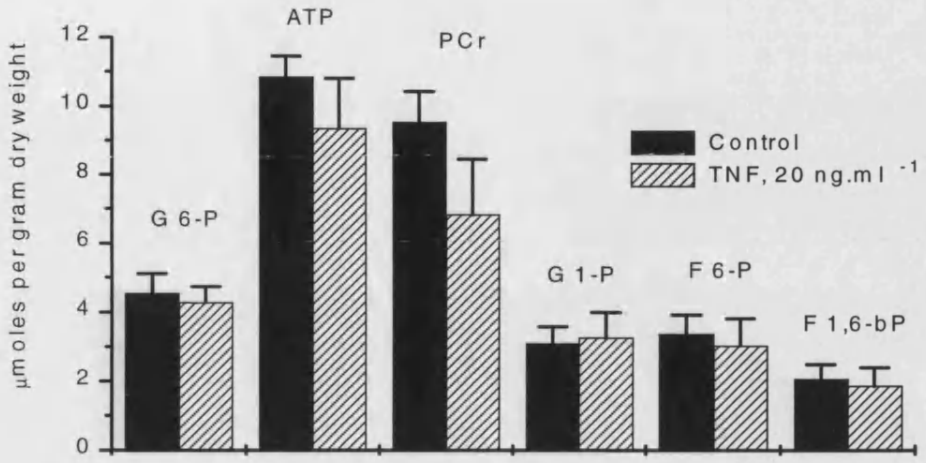


Figure 3.29. Effects of TNF and time on LVDP in the presence of normal glucose (11.6 mM) and high glucose (25 mM). 25 mM glucose perfusion commenced at A, TNF was added at B and recirculation began at C. * $P < 0.05$; TNF with high glucose vs. high glucose control ($n = 6$ for each point with high glucose). Significant differences with the normal glucose hearts have been omitted for clarity, see figure 3.25 for these.

3.5.4 End-point glycogen and products of the glycolytic pathway.

From the figure 3.30 it can be seen that addition of TNF did not alter end-point tissue levels of G-6-P, G-1-P, ATP, PCr, F-6-P or F-1,6-P under normal glucose, 11.6 mM conditions. Under high glucose conditions, TNF showed a tendency to reduce end point ATP levels. It can also be seen from this figure that none of the other products of the glycolytic pathway, mentioned above, were altered by TNF. When these values, are compared with the end-point ATP and PCr levels seen after global low flow ischaemia (table 3.1), then they are significantly lower in both normal glucose perfusion groups and high glucose perfusion groups both with and without TNF ($P < 0.05$). This is probably due to different protocols used with respect to the perfusion time. End point glycogen levels under both normal glucose and high glucose perfusion were also unaffected by TNF (table 3.2).

a)



b)

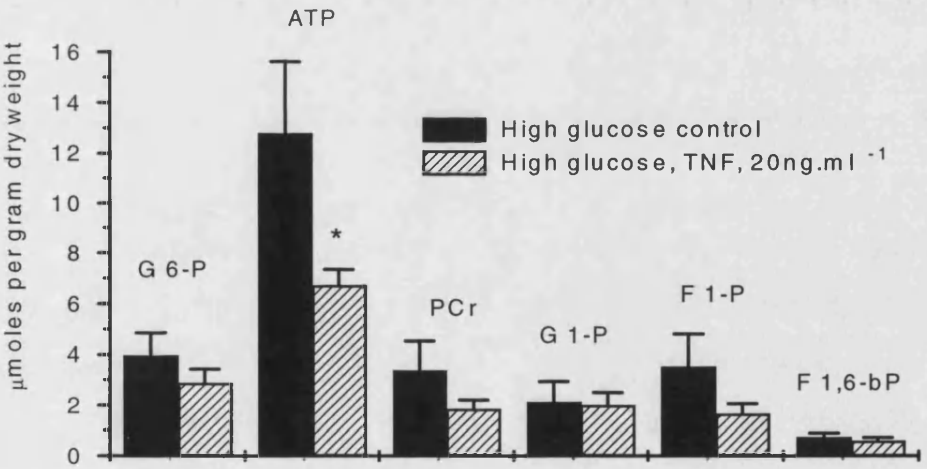


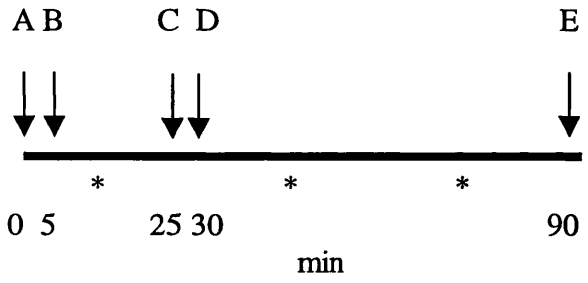
Figure 3.30. The above two graphs show the effects of TNF on end-point G-6-P, ATP, PCr, G-1-P, F 1-P and F-1,6-P for normal glucose, a (n = 6 - 9), and high glucose, b (n = 6 - 7), perfusion. * $P < 0.05$ TNF vs. control.

	Normal glucose perfusion		High glucose perfusion	
Treatment	Control	TNF, 20 $ng.ml^{-1}$	Control	TNF, 20 $ng.ml^{-1}$
End-point glycogen levels (μmoles of glucose equivilents per gram of tissue, dry weight)	68.0 ± 7.7	54.6 ± 8.8	47.08 ± 7.0	53.1 ± 7.4

Table 3.2. This table shows the end-point glycogen levels in both normal glucose perfused hearts, and high glucose perfused hearts, both in the presence and absence of TNF. (n = 6-10 for each group).

3.5.5 Actions of sphingomyelinase on cardiac function

Recent reports by Oral *et al.*, 1997 and Bozkurt *et al.*, 1998 have implicated sphingosine, a metabolite of the sphingomyelin pathway, in the early depression in cardiac function observed by TNF. So it was decided to see if the depression in cardiac function observed with TNF could be mimicked by exogenous addition of the enzyme SMase. The following protocol was used to investigate the actions of SMase on cardiac function:



A = initial perfusion B = antagonist or inhibitor C = TNF, 20 $ng.ml^{-1}$ or SMase, 0.001 $U.l^{-1}$, 0.003 $U.l^{-1}$ or 0.01 $U.l^{-1}$ D = recirculation E = end of perfusion

* show where Starling curves were performed.

Figure 3.31 shows the actions of different concentrations of SMase (0.001 $U.l^{-1}$, 0.003 $U.l^{-1}$ and 0.01 $U.l^{-1}$) on cardiac contractility. At all concentrations used SMase caused a marked negative inotropic effect. From this graph a concentration of 0.003 $U.l^{-1}$ which gave a maximal depression in function was chosen for all subsequent experiments. This depression in LVDP was also reflected in the Starling curves, figure 3.35. It can be seen from this that in the presnce of SMase there is a marked decrease in the Starling response of the left ventricle. SMase caused a coronary constriction, measured as an increase in

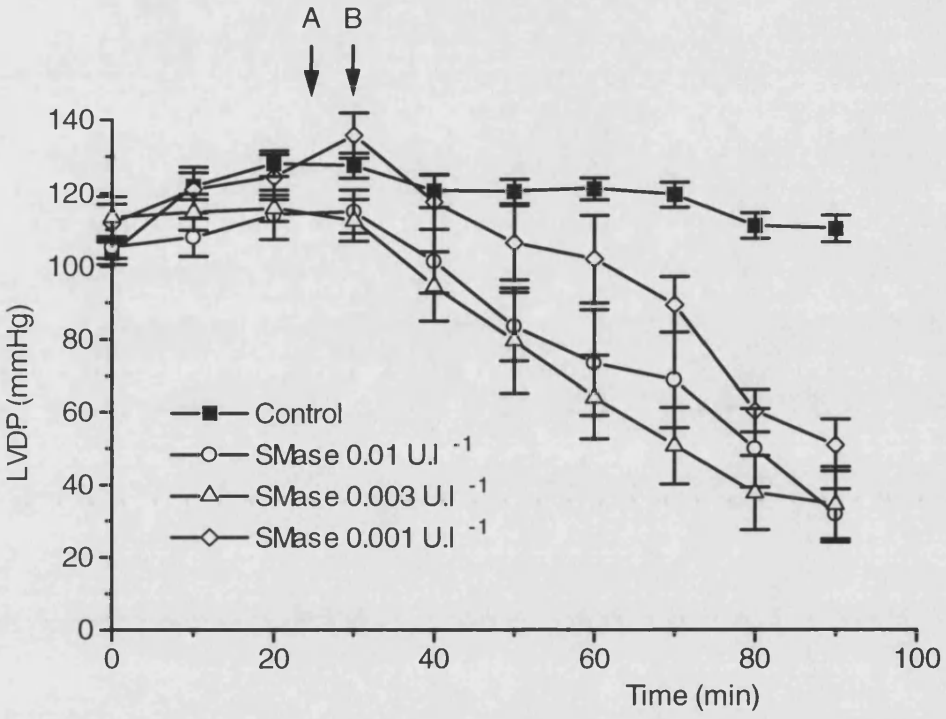


Figure 3.31. Effects of SMase on LVDP in isolated perfused hearts. SMase was added at A, and recirculation began at B. All concentrations used significantly reduced LVDP, but statistics have been omitted for clarity, figure 3.34 shows the statistical significance of the depression in LVDP for 0.003 $U.l^{-1}$.

CPP (increase in CPP 20 min after SMase: control, n = 7, vs. SMase, n = 8; 13 ± 5 mmHg vs. 75 ± 20 mmHg, P < 0.01). SMase did not alter heart rate (heart rate after 90 min: control vs. SMase; 285 ± 11 bpm vs. 273 ± 25 bpm, P = NS).

3.5.6 Effects of antagonists and inhibitors on changes in contractility caused by TNF and SMase under constant flow conditions.

To further investigate the depression in cardiac function observed with both TNF and SMase various antagonists and inhibitors were utilised. The results generated from these are shown below.

3.5.6.1 Nitro-L-arginine

NO has been implicated in TNF induced cardiac depression (Finkel *et al.*, 1992, Shultz *et al.*, 1995) therefore nitro-L-arginine was used to block NO production from isoforms of NOS (Gross *et al.*, 1990). Nitro-L-arginine, 100 μM, when included 20 min before addition of TNF, caused an intense coronary constriction (CPP before nitro-L-arginine vs. 20 min after nitro-L-arginine: 56 ± 2 mmHg vs. 126 ± 9 mmHg, P<0.001), which was apparent throughout the protocol. However, from figure 3.32 it is clear that inhibition of NOS did not ameliorate the decrease in contractility seen with TNF. In fact it is possible that nitro-L-arginine actually caused a slight potentiation of the cardiac depressant action of TNF. Heart rate was not altered by nitro-L-arginine (heart rate before nitro-L-arginine vs. after nitro-L-arginine: 289 ± 12 bpm vs. 290 ± 12 bpm, P = NS).

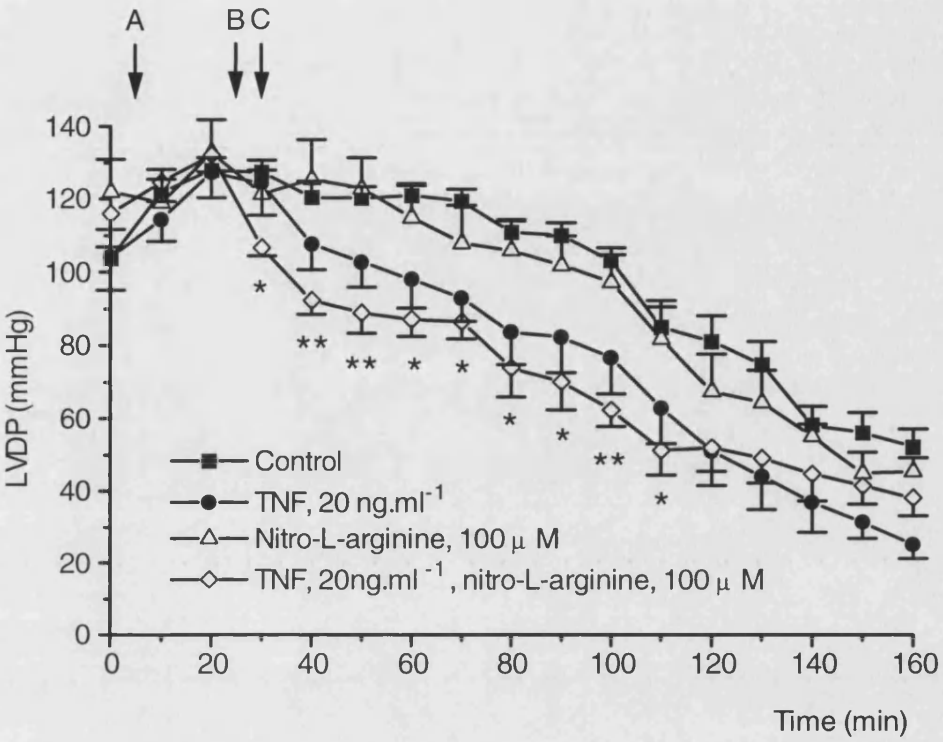


Figure 3.32. The depression in cardiac function observed with TNF is not altered by prior inclusion of nitro-L-arginine. Nitro-L-arginine was added at A, TNF was added at B and recirculation began at C. * $P < 0.05$, ** $P < 0.01$; TNF with nitro-L-arginine ($n = 8$) vs. nitro-L-arginine alone ($n = 4$).

3.5.6.2. N-oleoylethanolamine (NOE)

N-oleoylethanolamine (NOE) blocks ceramidase, the enzyme responsible for the conversion of ceramide to sphingosine (Sugita *et al.*, 1975, Oral *et al.*, 1997). In hearts where NOE, 1 μM, was included into the perfusate 20 min before addition of TNF, the negative inotropic effect of TNF was blocked (figure 3.33). NOE did not alter basal contractility (LVDP before NOE vs. after NOE: 110 ± 4 mmHg vs. 109 ± 4 mmHg, P = NS) or heart rate (heart rate before NOE vs. after NOE: 267 ± 10 bpm vs. 263 ± 9 bpm, P = NS). NOE also caused a significant attenuation of the depression in function seen upon addition of SMase (figure 3.34). This protection from SMase-induced depression in function with NOE, was also observed in the Starling response (figure 3.35), where SMase caused a significant reduction in Starling curves performed at 75 min which was attenuated by prior inclusion of NOE.

3.5.6.3 Indomethacin

The cyclooxygenase inhibitor indomethacin was used to block any potential breakdown of arachidonic acid from TNF induced PLA_2 activation. Indomethacin failed to alter the cardiac depression seen with TNF (LVDP after 90 min: TNF vs. indomethacin with TNF; 82 ± 9 mmHg ± 6 mmHg vs. 66 ± 6 mmHg, P = NS). Indomethacin had no effect on basal parameters of LVDP (LVDP before indomethacin vs. after indomethacin: 101 ± 4 mmHg vs. 105 ± 4 mmHg, P = NS) or heart rate (heart rate before indomethacin vs. after indomethacin: 263 ±4 bpm vs. 278 ±13 bpm, P = NS).

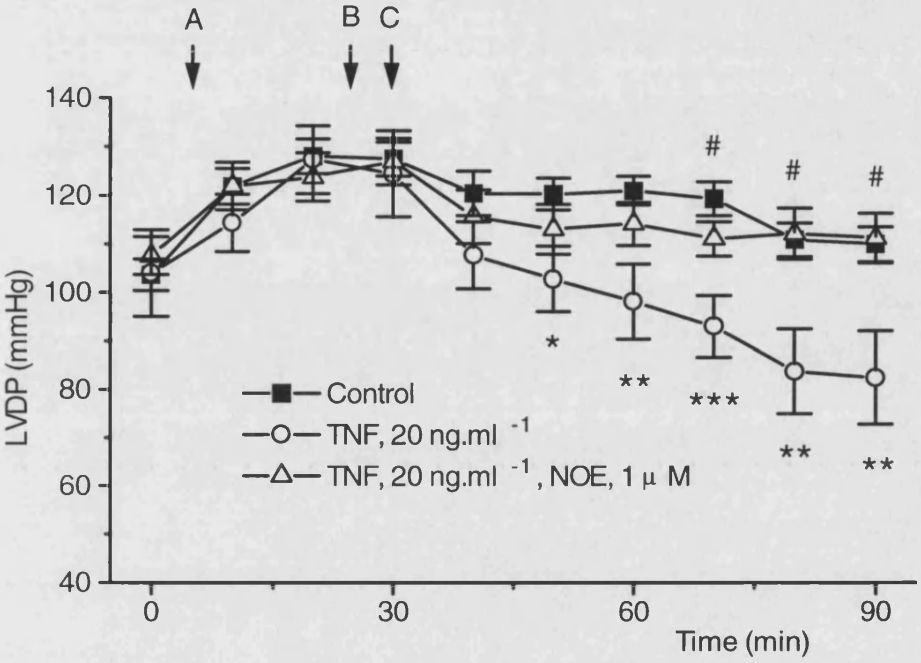


Figure 3.33. Effect of NOE on the depression in LVDP induced by TNF (n = 9). NOE was added at A, TNF perfusion began at B and recirculation was started at C. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; TNF vs. control. # $P < 0.05$; TNF and NOE vs. TNF alone.

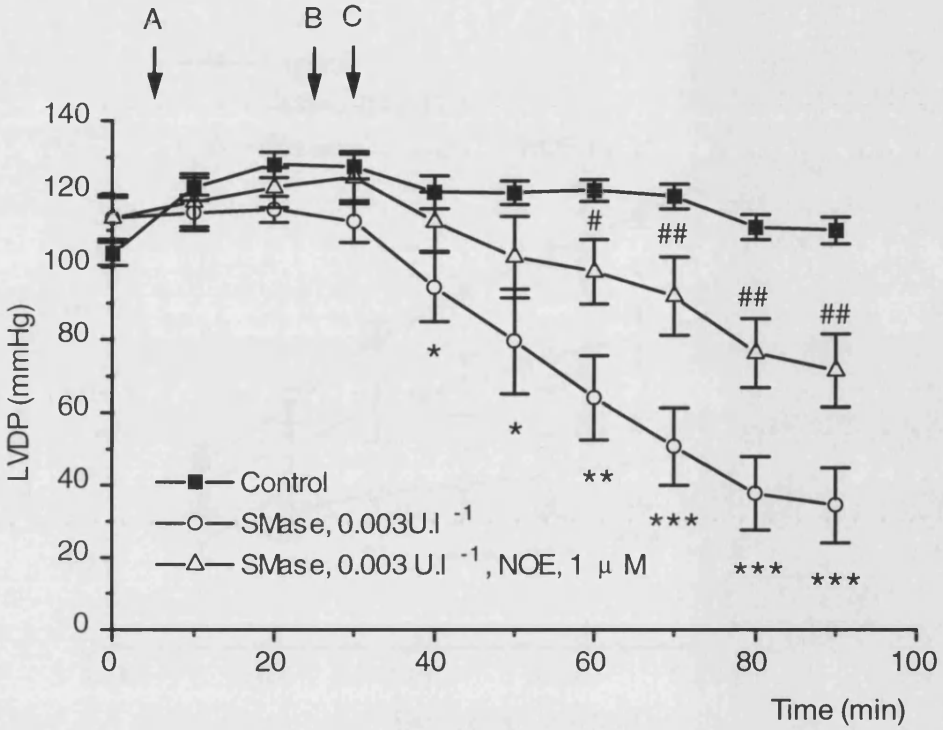


Figure 3.34. Effects of NOE (n = 8) on the action of SMase on LVDP in the isolated rat heart. Control data, n = 13. NOE was added at A, SMase was given at B and recirculation began at C. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; SMase alone vs. control. # $P < 0.05$, ## $P < 0.01$; SMase and NOE vs. SMase alone.

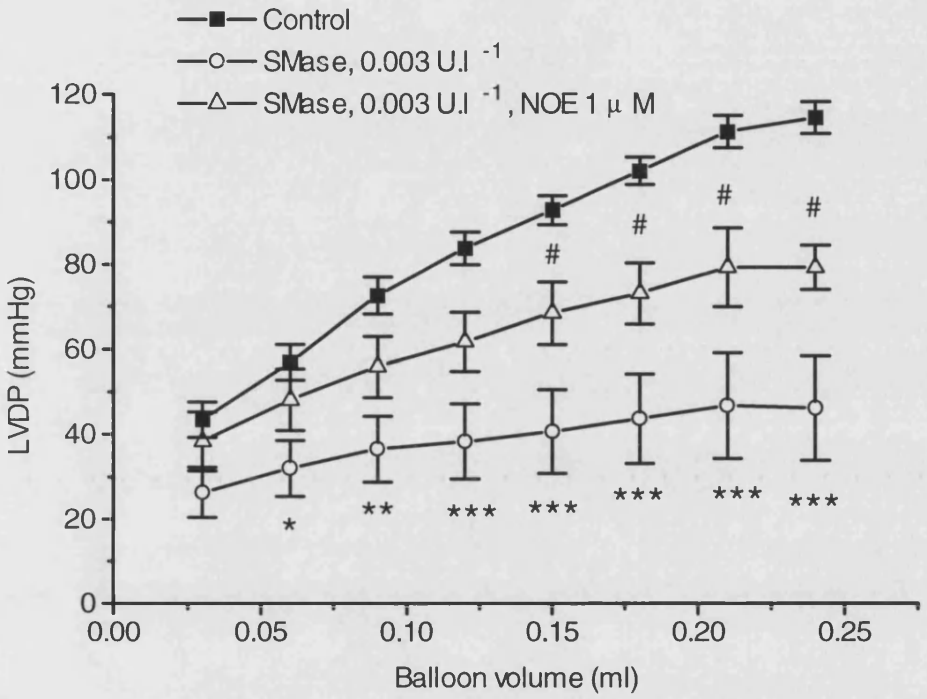


Figure 3.35. Effects of NOE on the action of SMase on the Starling response of hearts, measured at 75 min. SMase (n = 6), NOE (n = 8) and control data, n = 13. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; SMase alone vs. control. # $P < 0.05$, ## $P < 0.01$; SMase and NOE vs. SMase alone.

3.5.6.4. Bosentan

Due to the fact that TNF has been shown affect the whole heart by the release of endothelin-1 (Klemm *et al.*, 1995a; Hohfled *et al.*, 1995), potential actions of endothelin-1 were blocked by prior addition of the ET_A-ET_B-receptor antagonist bosentan (3 μM) into the perfusate. This did not block the TNF-induced alterations in cardiac function (LVDP after 90 min: TNF vs. bosentan and TNF; 82 ± 9 mmHg vs. 73 ± 6 mmHg, P = NS). Bosentan also did not alter the basal LVDP (LVDP before bosentan vs. after bosentan: 104 ± 2 mm Hg vs. 106 ± 2 mmHg, P = NS), or heart rate (heart rate before bosentan vs. after bosentan: 271 ± 9 bpm vs. 271 ± 6 bpm, P = NS).

3.5.7 Addition of recirculating perfusate from one heart to another

In order to see if the depression in LVDP seen upon administration of TNF was due to TNF-induced release, and subsequent build up, of a myocardial depressant substance into the recirculating perfusate, hearts were perfused with TNF as described above. After this the recirculating perfusate was taken from both a control heart and one perfused with TNF and passed through a separate heart for 10, 20 and 30 second periods. Upon application of the perfusate, from either TNF treated or control hearts, to a separate heart caused a decline in LVDP, however, there was no difference between the depression observed with the perfusate from control hearts, to that observed with the perfusate from TNF treated hearts. Change in LVDP with a 10 second perfusion was -15 ± 3 mmHg vs. –13 ± 3 mmHg (P = NS, n = 3) for perfusate from control and TNF treated hearts respectively.

Change in LVDP with a 20 second perfusion was -24 ± 5 mmHg vs. –17 ± 3 mmHg (P = NS, n = 3) for perfusate from control and TNF treated hearts respectively. Change in LVDP with a 30 second perfusion was –26 ± 6 mmHg vs. –15 ± 4 mmHg (P = NS, n = 3) for perfusate from control and TNF treated hearts respectively. See section 3.6.3 for the changes in CPP observed during this protocol.

3.5.8 Actions of recombinant rat TNF in the rat isolated perfused heart, under constant flow conditions

All of the experiments described above utilised recombinant human TNF to perfuse isolated rat hearts. Therefore, a series of experiments were completed in order to see if the cardiac depressant actions of recombinant human TNF could be reproduced with TNF derived from the relevant species of animal used in these studies. Experiments with rat TNF followed the same protocol as described with human TNF. As expected recombinant rat TNF, 20 $ng.ml^{-1}$, caused a decrease in LVDP which appeared similar to that seen with human TNF, and appeared of no greater severity (figure 3.36). This decline in developed pressure did not reach the levels of statistical significance observed with human TNF, however, the number of hearts used was much lower (n = 4). Interestingly, out of six hearts treated with recombinant rat TNF, one went into ventricular fibrillation and one became extremely arrhythmic to the extent that the developed pressure could not be accurately determined. This is in contrast to hearts perfused with human TNF, none of which went into severe arrhythmias or fibrillations, and suggests that recombinant rat TNF has an additional toxic effect when compared to human TNF in this model.

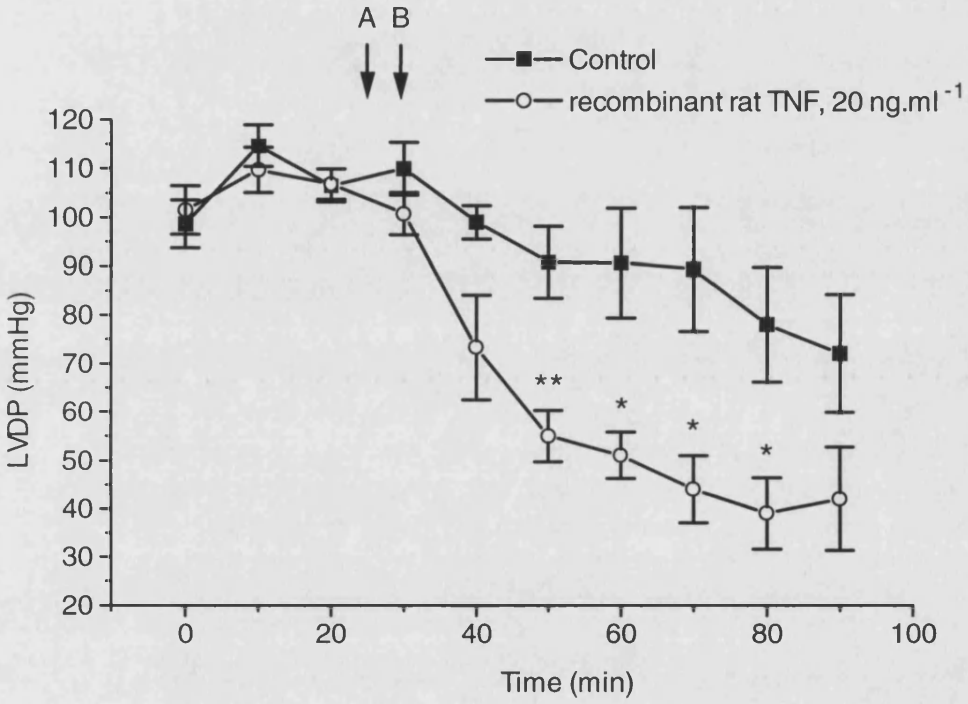


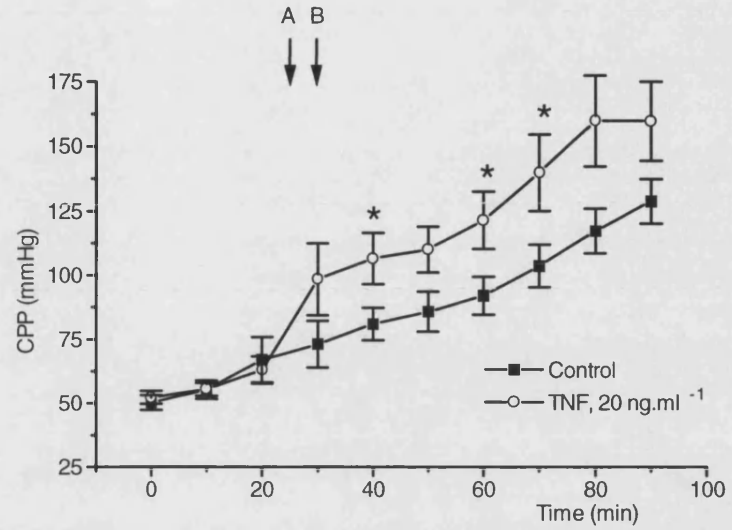
Figure 3.36. Action of recombinant rat TNF on LVDP in the rat isolated perfused heart. Recombinant rat TNF was added at A, and recirculation commenced at B. * $P < 0.05$, ** $P < 0.01$ recombinant rat TNF vs. control (control, n = 6; TNF, n = 4).

3.6 Effects of TNF in the coronary circulation

Whilst investigating the actions of TNF on cardiac contractility, using constant flow perfusion conditions, it was noted that TNF also caused an increase in coronary perfusion pressure (CPP), indicative of coronary constriction. A series of experiments were conducted to try to characterise this TNF-induced coronary constriction. See section 3.5 for protocol.

When switching from an open circuit to a recirculating system, CPP rose slowly but steadily throughout the 90 min experiment (figure 3.37). TNF addition into the Krebs buffer resulted in a rapid and sustained rise in CPP, which was indicative of a coronary constriction (figures 3.37 and 3.38). The experimental trace shown in figure 3.38 indicates that the majority of the rise in CPP occurred within 10 min of TNF addition. After this time CPP continued to rise in parallel with control hearts.

a)



b)

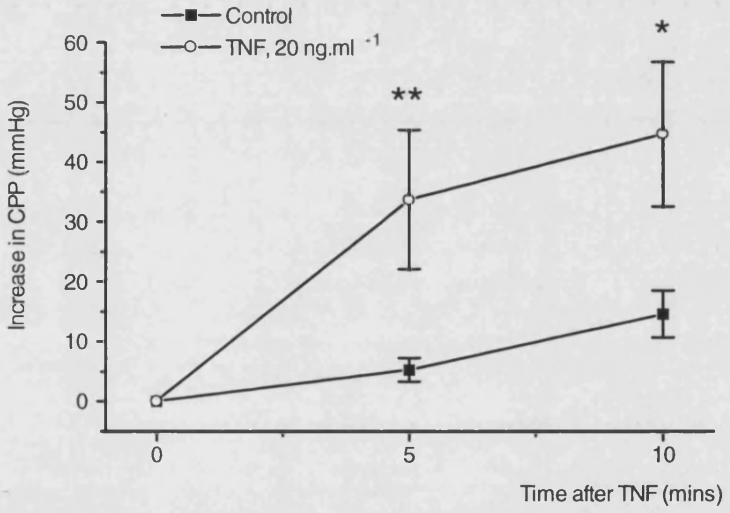


Figure 3.37. The above graphs show the rise in CPP, with time, and the effects of TNF in the isolated perfused heart under recirculating conditions. a) shows the actual CPP, whereas b) shows the increase in CPP after the addition of TNF. * $P < 0.05$, ** $P < 0.01$; TNF vs. control. Control data, n = 12, TNF data, n = 11.

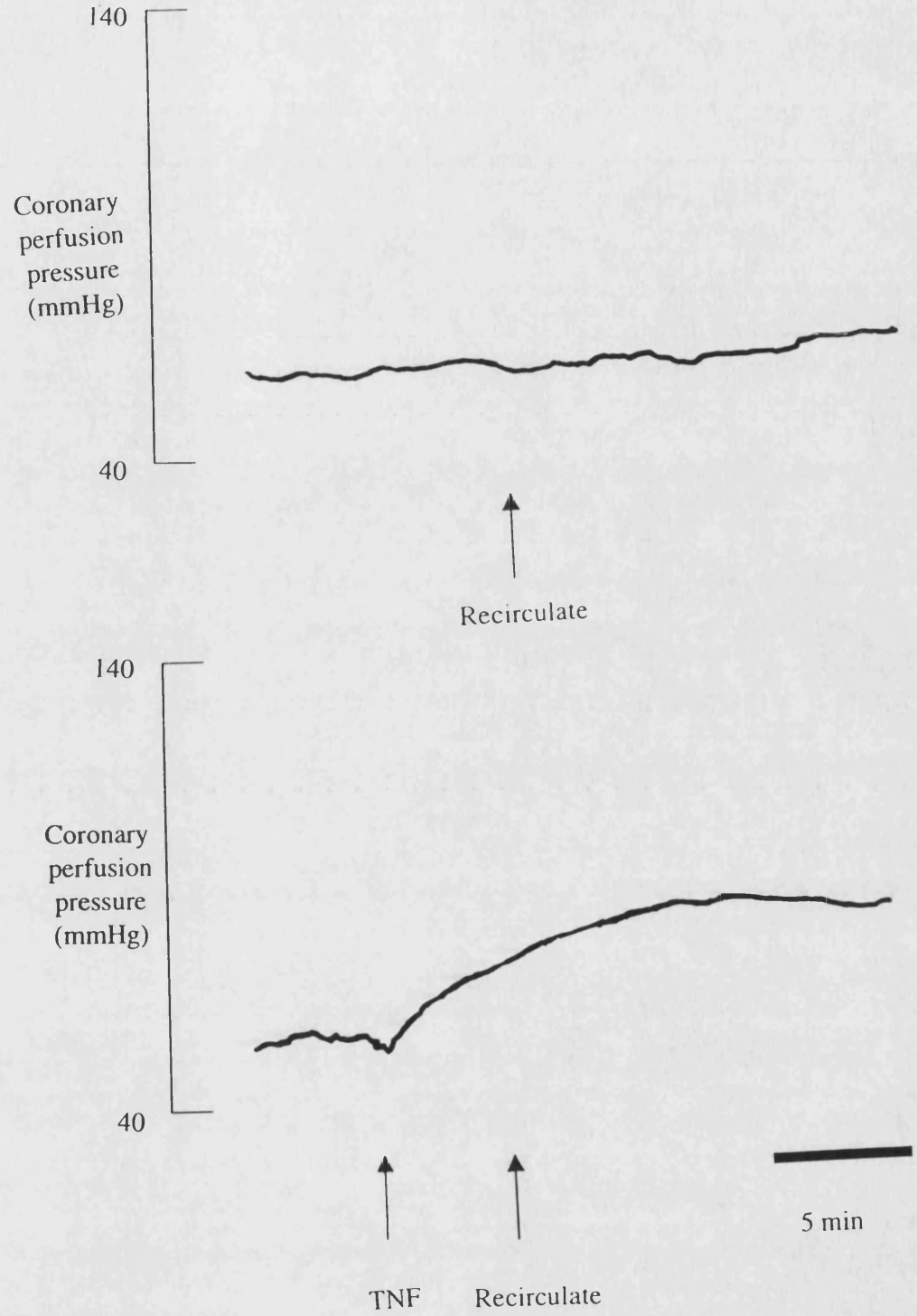


Figure 3.38. Typical experimental traces showing the coronary perfusion pressure changes in a control heart (a) and in a heart after TNF, 20 $ng.ml^{-1}$, administration (b). These traces are representative of 13 and 10 experiments respectively.

3.6.1 Effects of antagonists and inhibitors on the coronary constriction seen with TNF

As when characterising the actions of TNF on cardiac contractility, various antagonists and inhibitors were used. The results of these studies are presented below.

3.6.1.1 Indomethacin

The cyclooxygenase inhibitor indomethacin, 10 μM, which blocks the production of prostanoids from arachidonic acid (AA) breakdown, completely inhibited the TNF induced coronary constriction (figure 3.39). In the absence of TNF, indomethacin did not alter the increase in CPP observed over the 90 min perfusion period (figure 3.40). As mentioned before, indomethacin did not alter basal parameters of LVDP or HR.

3.6.1.2 NOE

As with the suppression in cardiac function, NOE, 1 μM, completely blocked the TNF induced rise in CPP (figure 3.39), but did not alter the basal coronary tone in the absence of TNF (CPP after 90 min: control (n = 13) vs. TNF (n = 6): 129 ± 9 mmHg vs. 113 ± 16 mmHg, P = NS). As mentioned before, NOE did not alter basal parameters of LVDP or HR.

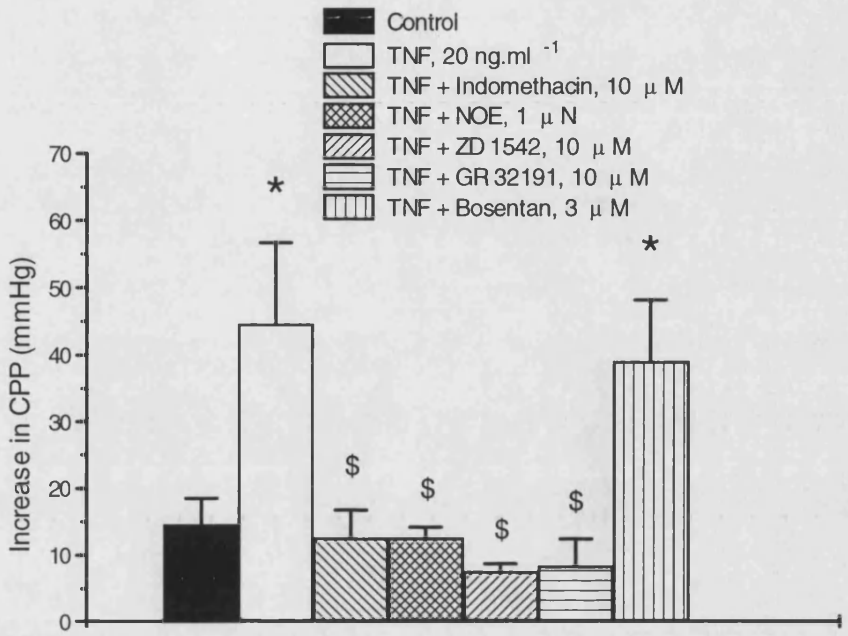


Figure 3.39. Effect of the antagonists indomethacin (n = 6), NOE (n = 9), ZD 1542 (n = 7), GR 32191 (n = 7) and bosentan (n = 7) on the TNF (n = 10) induced coronary vasoconstriction, 10 min after addition of TNF. Control in the absence of TNF n = 13. * P < 0.05 when compared with control data. $ P < 0.05 when compared with TNF data.

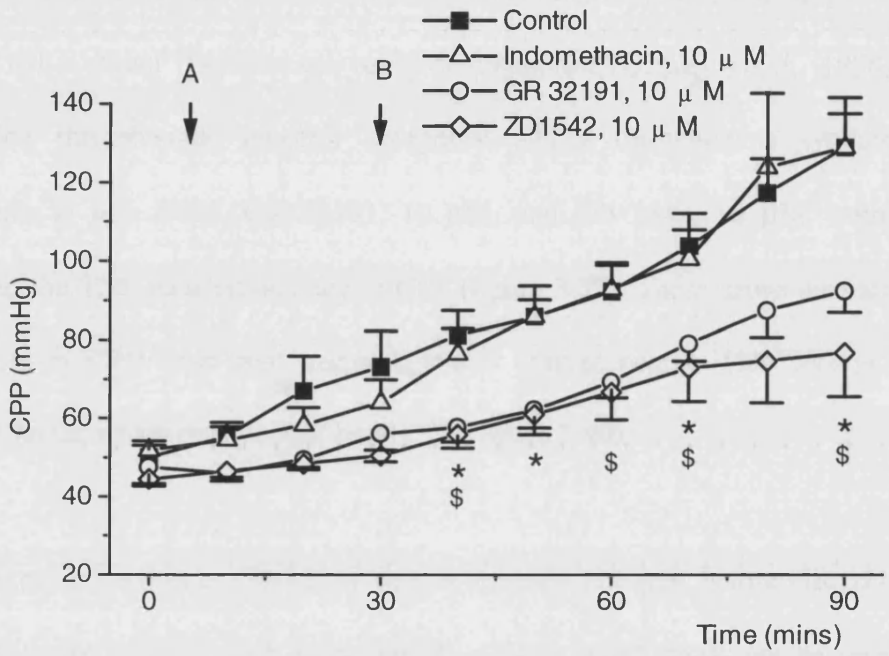


Figure 3.40. Effects of perfusion time (n = 12), indomethacin (n = 6), GR 32191 (n = 7) and ZD 1542 (n = 7) on CPP in the isolated perfused heart. Antagonists were added at A, recirculation began at B. * $P < 0.05$ GR 32191 vs. control. $ $P < 0.05$ ZD 1542 vs. control.

3.6.1.3 Thromboxane A_2 antagonists

Because indomethacin was shown to inhibit the TNF induced coronary constriction, it was decided to investigate the role of the vasoconstrictor prostanoid, thromboxane A_2, as a possible mediator of this vasoconstriction. To do this two different drugs were utilised. GR 32191 a direct thromboxane receptor antagonist (Lumley *et al.*, 1988) and ZD a combined thromboxane receptor antagonist and a thromboxane synthesis inhibitor (Brownlie *et al.*, 1993). GR 32191, 10 μM, and ZD 1542, 10 μM, both completely inhibited the TNF induced increase in CPP (figure 3.39). These drugs also attenuated the slow rise in CPP seen upon recirculation in control hearts. This was in contrast to indomethacin, which did not alter basal CPP (figure 3.40).

An unexpected action of GR 32191 was to decrease HR (HR before GR 32191 vs. after GR32191: 265 ± 12 vs. 205 ± 16 bpm, $P < 0.05$), which could not be reversed by the muscarinic receptor antagonist, atropine (10 μM, data not shown). This was accompanied by a slight increase in basal LVDP (LVDP before GR 32191 vs. after GR 32191: 96 ± 2 mmHg vs. 121 ± 8 mmHg, $P < 0.05$). This action was apparent in both the presence and absence of TNF. ZD 1542 did not alter either LVDP (LVDP before ZD 1542 vs. after ZD 1542: 98 ± 7 mmHg vs. 99 ± 6 mmHg, P = NS) or HR (HR before ZD 1542 vs. after ZD 1542: 272 ± 8 vs. 274 ± 9 bpm, P = NS).

3.6.1.4 Bosentan

Addition of bosentan, 3 μM, to the perfusate failed to alter the rise in CPP seen with TNF (figure 3.39). This concentration of bosentan was, however, able to block the coronary constrictor actions ET-1, 100 pM, in the coronary circulation (increase in CPP after 5 min perfusion: ET-1 vs. ET-1 in the presence of bosentan; 97 ± 6 mmHg vs. 13 ± 1 mmHg, $P < 0.05$). Bosentan did not alter CPP in the absence of TNF (CPP after 90 min: control vs. bosentan; 129 ± 9 mmHg vs. 114 ± 11 mmHg, P = NS). As mentioned before, bosentan did not alter basal levels of either LVDP or HR.

3.6.2 Actions of U 46619 on CPP and contractility

From the above presented results it is not clear whether the TNF-induced depression in cardiac function was independent of the observed coronary constriction, or if the depression in cardiac function was in part dependant on the observed coronary constriction. To address this possibility it was decided to mimic the coronary constriction with the stable thromboxane A_2 mimetic, U 46619, and to observe the effect of this vasoconstriction on contractile function.

Addition of U 46619, 30 nM, caused an immediate and significant increase in CPP (CPP increase 10 min after addition of U 46619 vs. control: 32 ± 8 mmHg vs. 15 ± 4 mmHg, $P < 0.05$). This increase in CPP was similar in magnitude to that seen with TNF, 20 $ng.ml^{-1}$

(45 ± 12 mmHg). However, U 46619 did not alter cardiac contractility (LVDP before U 46619 vs. LVDP after U 46619: 117 ± 3 vs. 121 ± 6 mmHg, P = NS).

3.6.3 Addition of recirculating perfusate from one heart to another

In order to see if the coronary constriction seen upon administration of TNF was due to TNF-induced release, and subsequent build up, of a coronary constrictor into the recirculating perfusate, hearts were perfused with TNF as described above. After this the recirculating perfusate was taken from both a control heart and one perfused with TNF and passed through a separate heart for 10, 20 and 30 second periods. There was no difference between the changes in coronary tone observed with the perfusate from control hearts, to that observed with the perfusate from TNF treated hearts. Change in CPP with a 10 second perfusion was -7 ± 5 mmHg vs. 0 ± 7 mmHg (P = NS, n = 3) for perfusate from control and TNF treated hearts respectively. Change in CPP with a 20 second perfusion was -8 ± 5 mmHg vs. –4 ± 4 mmHg (P = NS, n = 3) for perfusate from control and TNF treated hearts respectively. Change in CPP with a 30 second perfusion was –15 ± 11 mmHg vs. –5 ± 2 mmHg (P = NS, n = 3) for perfusate from control and TNF treated hearts respectively.

3.6.4 Actions of recombinant rat TNF in the coronary circulation of the rat isolated perfused heart, under constant flow conditions

Experiments described in this section (section 3.6) have all utilised recombinant human TNF. Therefore a series of experiments were completed in order to see if the coronary constrictory actions of recombinant human TNF could be reproduced with recombinant rat TNF. Experiments with rat TNF followed the same protocol as described with human TNF (section 3.6).

Recombinant rat TNF did not significantly alter CPP when compared with control hearts, as shown in figure 3.41, however it can be seen from this graph that the standard errors for each group were large. This was probably the result of low n-numbers of each group, especially the TNF treated group (n = 4). However, it can be seen from this graph that recombinant rat TNF did have a tendency to increase coronary tone, and a greater number of experiments would probably make this effect a significant one. Therefore, the actions of human TNF appeared to be mimicked by rat TNF.

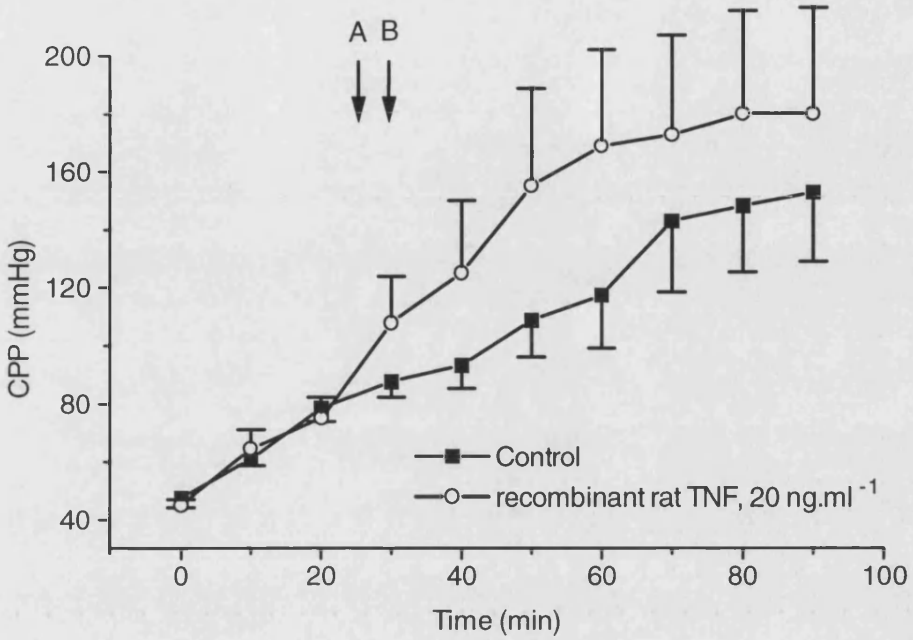
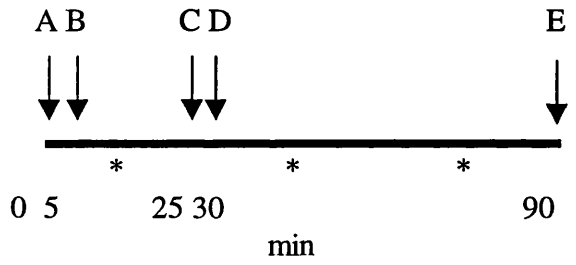


Figure 3.41. Effects of recombinant rat TNF on CPP in the rat isolated perfused heart under recirculating, constant flow conditions. TNF was added at A, and recirculation started at B (control hearts, n=6; TNF treated hearts, n=4).

3.7 Actions of TNF in the isolated rat heart under a constant head of pressure

From the experiments with U 46619 (section 3.6.2) it is clear that under constant flow conditions, coronary constriction of the magnitude seen with TNF does not adversely alter LVDP. However, under conditions where coronary flow can alter with coronary tone, such as with a constant pressure perfusion system, then such a constriction could alter substrate supply to the heart, and therefore contractile function. The diagram shown below describes the protocol followed:



A = perfusion under a constant head of pressure, B = NOE, 1μM, C = TNF 20 ng.ml^{-1}, D = recirculation, E = end of perfusion.

* show where Starling curves were performed.

As expected, upon recirculation in control hearts there was a slight decrease in coronary flow, which continued throughout the experiment (figure 3.42). As predicted from previous observations, prior addition of TNF caused a pronounced change in coronary

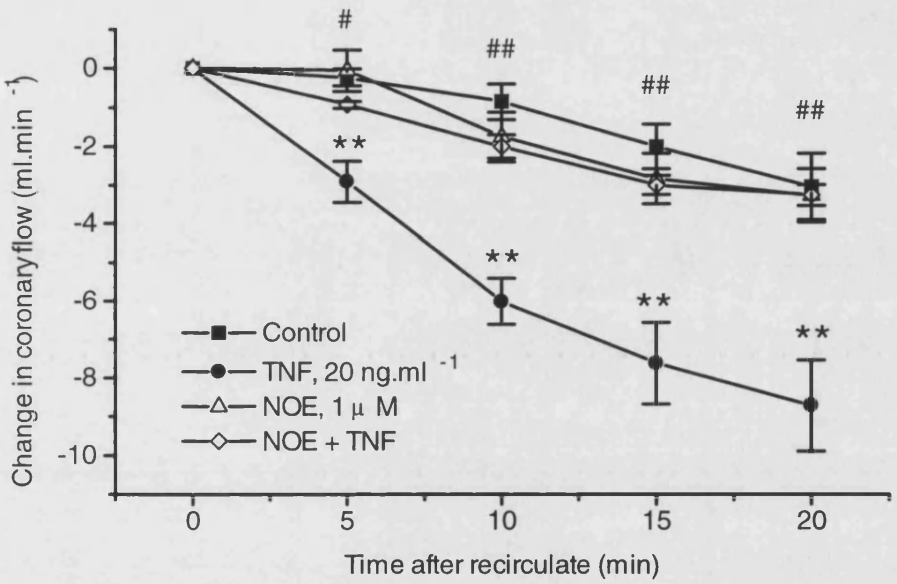


Figure 3.42. Effects of TNF and NOE alone and in combination on coronary flow in hearts perfused at constant pressure (n = 6 - 7) . ** $P < 0.01$ TNF vs. control, # $P < 0.05$, ## $P < 0.01$ NOE alone vs. TNF with NOE. Initial coronary flow at 0 min: 12.4 ± 0.6 ml.min^{-1}; 11 ± 1.2 ml.min^{-1}; 8.9 ± 1 ml.min^{-1} and 10.3 ± 2.4 ml.min^{-1} for control hearts, TNF treated, NOE treated and NOE with TNF treated hearts respectively.

tone, manifested by a decrease in coronary flow (figure 3.42). The changes in LVDP observed under constant pressure conditions followed a similar pattern to the changes observed under constant flow conditions, and a marked depression in function was seen in the presence of TNF (figure 3.43). However it is clear from this figure that the depression in cardiac function seen with TNF was more severe under constant pressure conditions than constant flow. Prior addition of NOE, 1 μM, blocked both the decrease in coronary flow (figure 3.42) as well as the depression in cardiac function observed with TNF (figure 3.44).

The more severe depression in cardiac function with TNF, observed under these conditions, was also apparent when performing Starling curves. As mentioned above, under constant flow conditions with TNF, a significant depression in the Starling response was not observed until the fifth and sixth Starling curves performed at 135 min and 165 min. Here, however, a marked depression in the Starling response was observed after 75 min (figure 3.44). Prior addition of NOE was able to block this depression in function (figure 3.45).

3.7.1 Effects of decreased temperature on cardiac function

Whilst examining the action of TNF in isolated hearts perfused with TNF, under a constant head of pressure, TNF caused a vasoconstriction and the reduction in flow caused the temperature of the heart dropped to below 37 °C, as measured by a temperature probe inserted into the right ventricle. This drop in temperature was despite

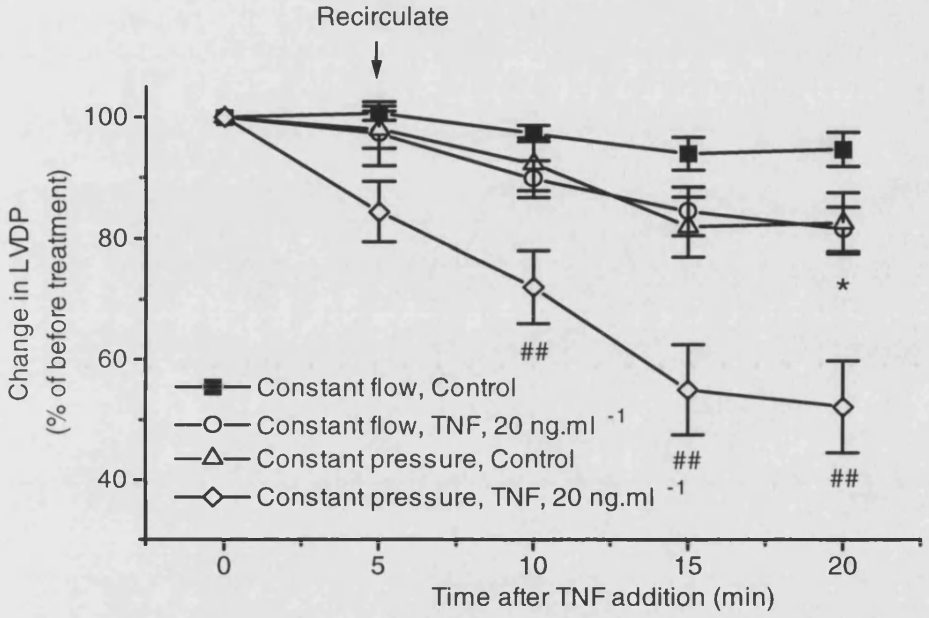


Figure 3.43. Effects of TNF on LVDP in hearts perfused under constant pressure and constant flow conditions (n = 6 - 7). Change in LVDP is shown as a percentage of before TNF addition to allow comparison between the two groups. * $P < 0.05$ TNF vs. control when perfused under constant flow conditions. \$ $P < 0.05$, \$\$ $P < 0.01$, TNF under constant flow conditions vs. TNF under constant pressure conditions. Where initial LVDP at 0 min: 122 ± 4 mmHg; 126 ± 6 mmHg; 101 ± 6 mmHg and 106 ± 3 mmHg for constant flow control and TNF treated heart and constant pressure control and TNF treated hearts respectively.

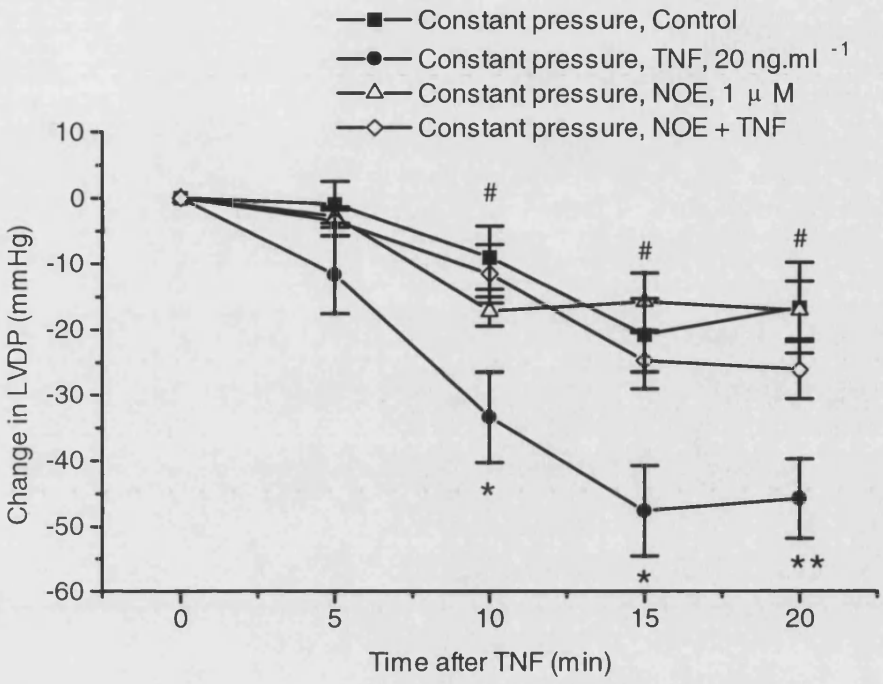


Figure 3.44. Effects of NOE on the depression in cardiac function seen with TNF under constant pressure conditions. Shown as changes in LVDP after TNF administration (n = 6 - 7). * $P < 0.05$, ** $P < 0.01$ TNF vs. control. # $P < 0.05$ NOE vs. TNF with NOE. Where LVDP at 0 min for NOE treated controls and NOE with TNF treated animals: 107 ± 6 mmHg and 100 ± 5 mmHg respectively.

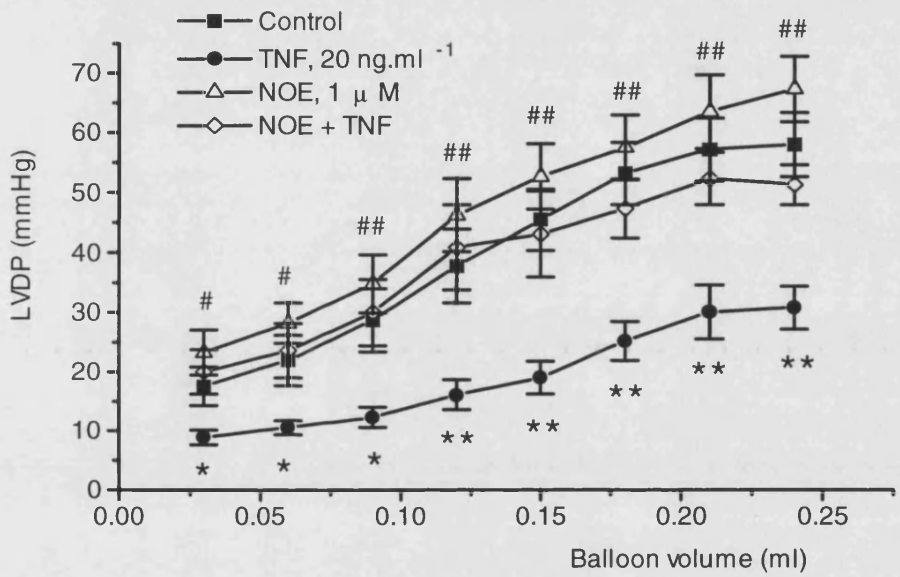


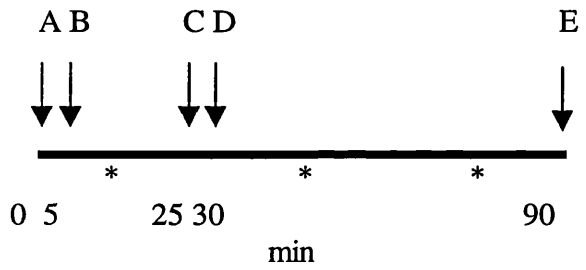
Figure 3.45. Starling curves in hearts perfused under a constant head of pressure, 50 min after TNF addition. Control hearts (n = 7), TNF, alone (n = 7), NOE alone (n=6) and TNF with NOE. * $P < 0.05$, ** $P < 0.01$, TNF vs. control. # $P < 0.05$, ## $P < 0.01$, NOE alone vs. TNF with NOE.

the fact that the hearts were surrounded by a heated water jacket. For this reason the effects of a drop in temperature on cardiac function was investigated.

A drop in temperature of the Krebs buffer from 37 °C to 33 °C caused a decrease in heart rate, from 286 ± 9 bpm to 201 ± 9 bpm ($n = 4$, $P < 0.05$) and a concomitant increase in LVDP, from 123 ± 2 mmHg to 133 ± 5 mmHg ($n = 4$, $P < 0.05$).

3.8 Sphingosine in the isolated perfused rat heart

The experiments described above present evidence implicating the sphingoid base, sphingosine, as an essential mediator of the early depression in cardiac function and an important mediator of the coronary constriction seen upon TNF administration to isolated hearts. By this rational, some, if not all, of the actions of TNF described above, should be mimicked by exogenous addition of sphingosine. The following protocol was used to examine the actions of sphingosine in the isolated rat heart:



A = initial perfusion, B = antagonists, C = sphingosine, 0.5 μM, 1 μM, 3μM or 10 μM, D = recirculation, E = end of perfusion.

* show where Starling curves were performed.

Addition of sphingosine (0.5 - 10 μM) to the isolated perfused hearts resulted in profound changes in the parameters of LVDP, left ventricular end diastolic pressure (LVEDP) and CPP. Figure 3.46 shows typical experimental traces from hearts perfused with 1μM, 3 μM and 10 μM sphingosine. It can be seen from these traces that hearts perfused with 10 μM sphingosine showed a dramatic increase in LVEDP, which was accompanied by an equally dramatic fall in LVDP. The actions of 10 μM sphingosine were so severe that all subsequent experiments used 3 μM sphingosine. Lower concentrations of sphingosine

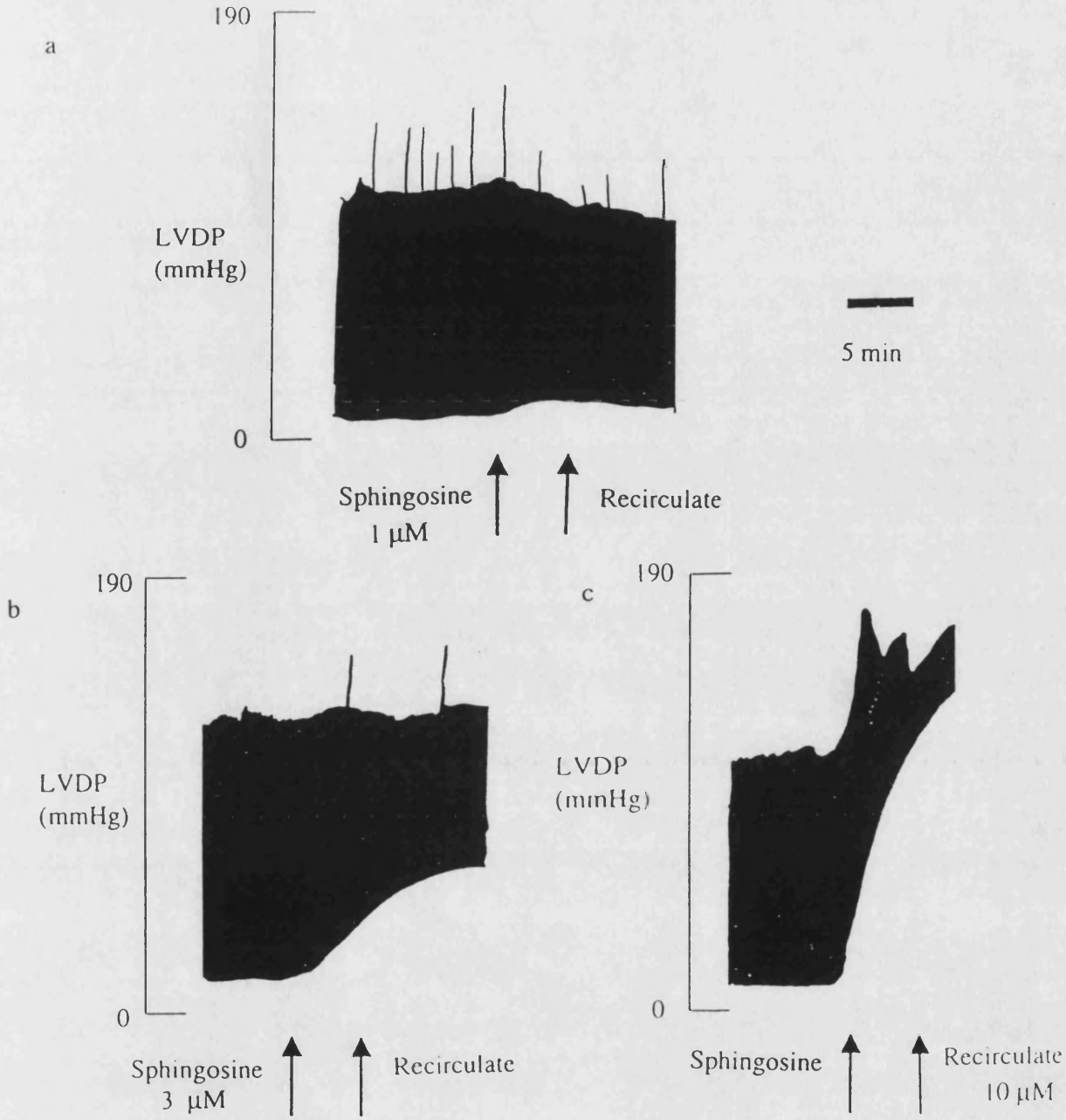


Figure 3.46. Typical experimental traces showing changes in LVDP upon application of sphingosine, 1μM (a), 3 μM (b) and 10 μM (c). These traces are representative of 6, 7 and 2 experiments respectively.

followed the same pattern, but to a lesser degree, figure 3.46. Figure 3.47 shows the response of hearts to sphingosine, 3 μM and 10 μM, when contractility was measured using both a hook in the apex of the heart and an intraventricular balloon to measure contractility. Interestingly, it is shown that although an increase in LVEDP is observed upon administration of sphingosine, this was not observed as in increase in the baseline hook tension. Upon perfusion with sphingosine, 3μM, there was an increase in CPP (figure 3.48), which followed slightly slower kinetics to the rise in CPP seen with TNF, plateauing after 15 min, as opposed to 10 min with TNF. Also, unlike with TNF, this coronary constriction was not altered by prior addition of indomethacin, 10 μM or ZD 1542 μM (figure 3.48).

In studies undertaken by Candela *et al*,. 1991, TNF was shown to cause the release of prostaglandin E_2 from fibroblasts, this was an action which showed remarkable synergy when in the presence of low concentrations of exogenous sphingosine (0.5 μM - 10 μM). Both prostaglandin E_2 and thromboxane A_2 are downstream metabolites of cyclooxygenase induced breakdown of arachidonic acid. For this reason the actions of TNF on cardiac contractility and coronary tone were observed in the presence of a low concentration of sphingosine, 0.5 μM.

When both a low concentration of sphingosine, 0.5 μM, and TNF, 20 $ng.ml^{-1}$, were perfused together through hearts, sphingosine did not potentiate any of the observed actions of TNF on LVDP or CPP (LVDP after 90 min, TNF vs. TNF with sphingosine: 82

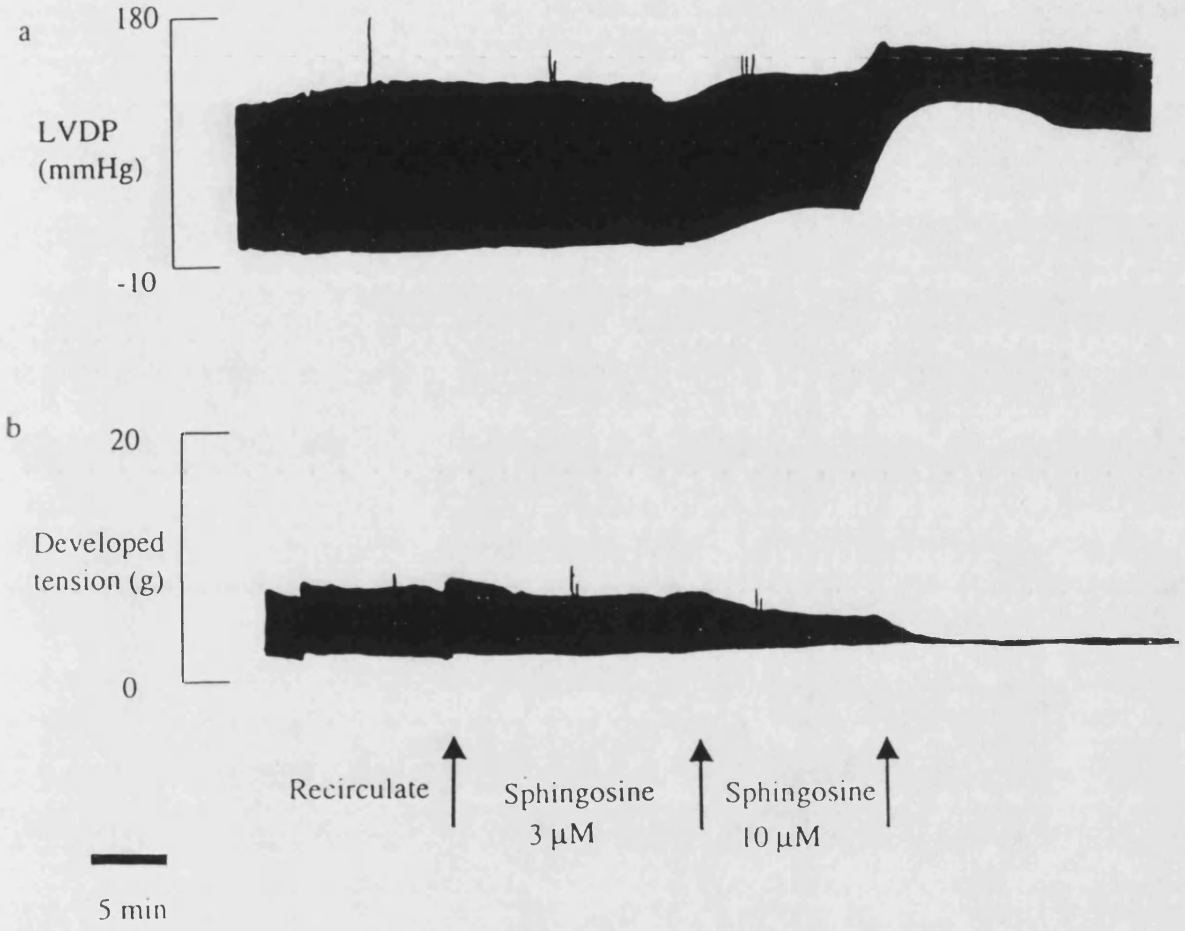


Figure 3.47. A typical experimental trace showing the effect of sphingosine, 3 μM and 10 μM in this isolated perfused rat heart. Contractility in this heart was simultaneously measured using a hook in the apex of the heart (a), and an intraventricular baloon (b).

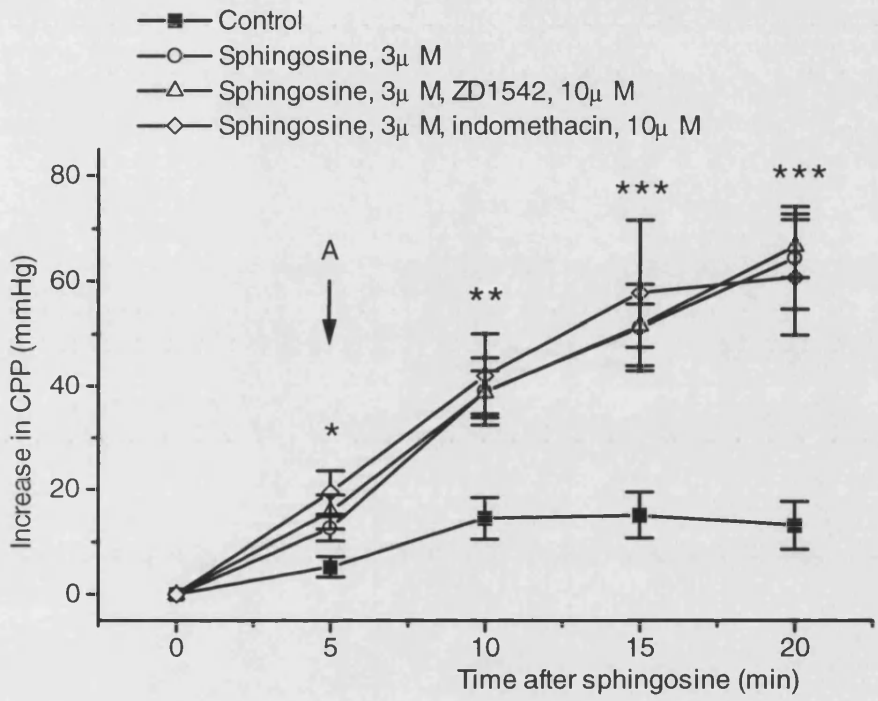


Figure 3.48. Effects of indomethacin (n = 4) and ZD 1542 (n = 4) on actions of sphingosine (n = 7) on CPP. Recirculation began at A. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; sphingosine vs. control.

± 10 mmHg vs. 75 ± 12 mmHg, P = NS. CPP after 90 min, TNF vs. TNF with sphingosine; 160 ± 15 vs. 164 ± 18 mmHg, P = NS).

Section 4 Discussion

During this study, various investigations have been carried out using the rat isolated perfused heart with the objective of describing the cardiac actions of TNF. It has been investigated whether TNF alters the recovery of hearts after a brief acidotic challenge. TNF has been shown to block insulin-stimulated glycogen synthesis without altering insulin-stimulated glucose uptake. Ceramide has been investigated as a potential mediator of this response. Contractile activity of hearts during low flow ischaemia under low glucose conditions was shown to be a increased by TNF. In hearts perfused under normal glucose conditions TNF acutely depressed contractile function, an effect which was investigated using pharmacological probes. In addition to this, TNF was shown to increase the tone of the coronary circulation and attempts to characterise these effects of TNF with pharmacological tools were made. It was also shown that under conditions where coronary flow could alter with coronary tone, the observed coronary constrictor action of TNF could indirectly depress cardiac contractility and synergise with the direct cardiac depressant actions of TNF. When characterising both the contractile and coronary effects of TNF, particular attention was paid to the sphingomyelin metabolite, sphingosine. As sphingosine was strongly implicated as a mediator of both the direct negative inotropic effects and the coronary effects of TNF, the actions of sphingosine in the isolated perfused heart were also investigated.

In this section, each of these investigations will be discussed as individual studies in their own right.

4.1 Preliminary experiments

Preliminary experiments were conducted in order to define conditions under which to investigate the actions of TNF. It was decided to use a recirculating mode of perfusion as opposed to a non-recirculating mode, in order to reduce the amount TNF needed for each individual experiment. Therefore, it was necessary to describe a recirculating model with which to perfuse isolated hearts, and to assess the viability of these hearts under these conditions. 50 ml was used as the total recirculating volume. The results presented in the last section (section 3,1, figure 3.1) show that although the process of recirculation slightly reduced contractility, measured as developed tension using a hook in the apex of the heart, hearts perfused under recirculating conditions were still viable after at least 2 hours perfusion. This reduced contractility was probably a result of the build up of metabolites released from the heart during normal function.

It should be noted that upon addition of drugs to a recirculating system, any secondary release of substances could build up in the recirculating perfusate, and so any observed actions of these released substances could be confused with the direct actions of the drug. Another potential problem with a recirculating mode of perfusion is that after addition of a drug it is impossible to wash that drug out without switching back to a non-recirculating mode of perfusion. Thus the wash out of metabolites released from normal heart function, could effect measured parameters of heart function, and so any effects of wash out of drugs may not simply be due to the loss of the drug in question from the system.

Despite these potential problems, a recirculating mode of perfusion, using a total volume of 50 ml was used to study the actions of TNF in the rat isolated perfused heart.

The second objective of these preliminary experiments was to find a concentration of TNF to use. A concentration of 20 $ng.ml^{-1}$ was chosen for two reasons. Firstly similar concentrations of TNF have been observed in sera from animals during experimental sepsis (Tracey *et al.*, 1987). Secondly, other investigators have used a concentration of 20 $ng.ml^{-1}$ during studies into the cardiac actions of TNF (Schulz *et al.*, 1995). Indeed, upon administration of TNF, 20 $ng.ml^{-1}$, a negative inotropic effect was observed within 15 min of administration, which was evident for the remainder of the experimental procedure. A higher concentration of TNF, 100 $ng.ml^{-1}$, failed to potentiate this depressed cardiac function. Therefore, it was decided that TNF at the concentration of 20 $ng.ml^{-1}$ would be used in subsequent experiments.

4.2 TNF and cardiac acidosis

It has been shown that at low pH levels the trimeric structure of TNF is relaxed, and it has been suggested that in this state TNF can associate with hydrophobic regions of the plasma membrane, and form sodium (Na^+) permeable channels (Kagan *et al.*, 1992; Baldwin *et al.*, 1996). During cardiac ischaemia a build up of protons occurs, both from ATP hydrolysis and release of lactate from the heart, resulting in a decrease in intracellular pH. Intracellular pH has been reported to reach values as low as 6.0 during such ischaemic episodes (Kolacassides *et al.*, 1996). Due to the associated increased circulating levels of TNF in ischaemic heart disease (Vaddi *et al.*, 1994), as well as the realisation that TNF release can be observed with experimental ischaemia/reperfusion injury (Squadrito *et al.*, 1993; Colletti *et al.*, 1990), it was decided to investigate whether TNF could alter the recovery of hearts after an experimental acidotic challenge. Any increase in Na^+ influx across the plasma membrane due to TNF would contribute to the increase in intracellular Na^+ observed during ischaemia (Pike *et al.*, 1990). This, via the action of the Na^+/Ca^{2+} ion exchanger, in turn could contribute to the detrimental Ca^{2+} overload often associated with ischaemia (Tani & Neely, 1989). Indeed, studies have shown that the protective effects of adenosine during ischaemia/reperfusion injury are associated with a decreased release of TNF from the heart (Meldrum *et al.*, 1998). In addition to this, neutralisation of TNF released during ischaemic injury can afford protection (Squadrito *et al.*, 1993). This protection could be explained if, under the acidotic conditions expected during ischaemia, TNF formed these Na^+ permeable ion channels.

In this study, acidosis was achieved by omission of sodium bicarbonate from the Krebs buffer solution, and subsequent addition of small quantities of sodium bicarbonate until the desired pH was achieved. An acidotic challenge, pH either 5.5 or 6.2 for 5 min, resulted in very reproducible changes in parameters of developed tension and CPP, as well as very reproducible recovery of hearts after such challenges. Prior addition of TNF, 5 min before acidosis, did not significantly depress the recovery of hearts after acidosis. Under both acidotic pH values, prior inclusion of TNF may have slightly reduced the contractility during recovery period, however this was not statistically significant, and probably represented an aspect of the early direct negative inotropic effect of TNF discussed later (section 4.5).

Baldwin *et al.* (1996) showed that TNF associates with membranes at a very low pH of 4. Therefore, the lack of effect of TNF described above could have been because the acidotic challenge was not severe enough. This does not eliminate the formation of Na^+ permeable ion channels by TNF as a potential deleterious action of TNF during ischaemia/reperfusion injury, however, it is unlikely to be a major factor in determining the outcome of an ischaemic episode. It should, however, be noted that although pH values of 6 have been reported during ischaemia (Kolacassides *et al.*, 1996), it is likely that under such conditions pools of proton accumulate at local sites, potentially decreasing local pH to very low values not observed with current techniques.

4.3 Insulin, TNF, ceramide and the low glucose recirculating heart

The aim of these investigations was to observe the actions of insulin in the isolated perfused heart under low glucose, 2 mM, conditions and to see if any of its actions could be altered by TNF. Under these substrate limiting, low glucose, conditions one would expect that the actions of insulin would be more important for cardiac function. The findings of this study can be summarised as follows: under low glucose conditions, developed tension declined at a faster rate when compared with perfusion under normal glucose, 11.6 mM, conditions. Under low glucose conditions, insulin slightly improved developed tension when added both at the start of the experiment or 15 min after recirculation. On neither occasion was this slight improvement in contractility observed in the presence of TNF. Insulin caused a marked stimulation of glucose uptake, as measured by a decrease in the glucose concentration within the recirculating Krebs buffer. Insulin also increased end point glycogen levels, suggesting stimulation of either glycogen synthesis, inhibition of glycogen breakdown or both of these. TNF, when added either before or after insulin did not alter insulin-stimulated glucose uptake, however in both circumstances TNF attenuated insulin-stimulation of end-point glycogen levels. TNF did not alter the breakdown of glycogen under zero glucose conditions. C_2-ceramide mimicked these actions of TNF, blocking the increase in end point glycogen levels without altering insulin-stimulated glucose uptake. Addition of the protein phosphatase inhibitor, okadaic acid, at a concentration where inhibition is selective for the PP-2A family of phosphatases including ceramide-activated protein phosphatase (CAPP) (Dobrowsky & Hannun, 1992), blocked the

actions of ceramide on insulin-stimulated end point glycogen levels. Okadaic acid caused only a slight attenuation of the TNF-induced depression in insulin-stimulated glycogen synthesis, although this was not statistically significant.

As mentioned in the introduction there is a great deal of interest in the mechanisms behind TNF-induced insulin resistance since the realisation that TNF could mediate the insulin resistance observed during obesity-induced diabetes and NIDDM (Hotamisligil *et al.*, 1993). Although there is much discrepancy between studies, much of the research in this area has focused on the theory that TNF alters the actions of insulin at a proximal site with respect to the insulin receptor signal (Feinstein *et al.*, 1993; Hotamisligil *et al.*, 1994a; Hotamisligil *et al.*, 1994b; Kanety *et al.*, 1995; Peraldi *et al.*, 1996; Hotamisligil *et al.*, 1996; Wang *et al.*, 1998). It has been shown that TNF can cause changes in the ability of the insulin receptor to undergo autophosphorylation accompanied by a decrease in the phosphorylation of IRS-1 (Feinstein *et al.*, 1993; Hotamisligil *et al.*, 1994a). It has also been suggested that an altered serine phosphorylation state of IRS-1 induced by TNF could turn it into an inhibitor of the insulin receptor (Kanety *et al.*, 1995; Peraldi *et al.*, 1996; Hotamisligil *et al.*, 1996). Various different time courses have been reported to be required for TNF-induced insulin resistance to become apparent from one hour (Feinstein *et al.*, 1993; Begum & Ragolia, 1996) to a few hours (Lang *et al.*, 1992; Peraldi *et al.*, 1996), or even a few days (Hotamisligil *et al.*, 1996; Wang *et al.*, 1998) incubation time with TNF.

The results presented herein show differential effects of TNF on different aspects of insulin action in the isolated perfused heart under low glucose conditions, whereby TNF attenuated insulin-stimulated end point glycogen levels without altering insulin-

stimulated glucose uptake. These differential effects suggest that a direct or indirect, via IRS-1, inhibition of the insulin receptor is not the mechanism responsible for TNF-induced alterations in insulin action in this model. If inhibition of the insulin receptor was to occur, then both insulin-stimulated glucose uptake and insulin-stimulated glycogen synthesis would be affected, instead only end point glycogen levels were altered by TNF. The selective action of TNF on insulin-stimulated glycogen synthesis suggests a mechanism of action at a site down stream of the insulin receptor. In part these studies agree with a recent report by Begum & Ragolia (1996), who showed that TNF could have differential actions on insulin action in a skeletal muscle cell line. It was shown that over 60 min incubation with TNF, 10 $ng.ml^{-1}$, was required to inhibit insulin-stimulated glucose uptake, however, the enzyme responsible for glycogen synthesis, GS, could be inhibited by only 10 min incubation with TNF. This effect on glycogen synthesis was accompanied by inhibition of insulin-stimulated activation of another glycogen regulatory enzyme, PP-1. These authors suggested that the effects of TNF on insulin-stimulated glycogen synthesis were more sensitive than the effects observed on insulin-stimulated glucose uptake (Begum & Ragolia, 1996). Maybe in my model, longer exposure to TNF would have decreased the ability of insulin to stimulate glucose uptake.

In my studies it was shown that the cell permeable analogue of ceramide, C_2-ceramide, could mimic the effects of TNF on insulin-stimulated glycogen synthesis, without altering insulin-stimulated glucose uptake. Again these data are similar to those of Begum & Ragolia (1996) as well as those of Peraldi *et al.* (1996) who showed that the actions of TNF with respect to insulin sensitivity could be reproduced by cell permeable ceramide analogues or exogenous addition of SMase. Begum & Ragolia

(1996) actually showed that treatment of their skeletal muscle cell line with TNF caused an increase in cellular ceramide with a concomitant decrease in sphingomyelin. The only difference between these studies and mine was that both of these studies showed TNF-induced disruptions of insulin-stimulated glucose uptake. Peraldi *et al.* (1996) showed that both TNF and ceramide could act to inhibit the insulin receptor and subsequent phosphorylation of IRS-1. This does not appear to be the case in my studies for the reasons discussed above.

Inclusion of okadaic acid into my system completely blocked the inhibitory actions of ceramide on the insulin-induced glycogen synthesis, however although okadaic acid did show a tendency to block the inhibitory effects of TNF on insulin-induced glycogen synthesis, this did not achieve statistical significance. Therefore, although ceramide may play a role in this action of TNF, it is clear that other mechanisms for TNF-induced attenuation of insulin-stimulated glycogen synthesis must exist. From my studies it is unclear what these other mechanisms may be.

Okadaic acid, at the concentrations used, is a specific inhibitor of the PP-2A family of phosphatases (Cohen *et al.*, 1989) of which CAPP is one (Dobrowsky & Hannun, 1992). As mentioned above the effects of ceramide on insulin action were blocked by okadaic acid, therefore it appears that the observed actions of ceramide on insulin-stimulated end point glycogen levels involve activation of CAPP. PP-2A phosphatases, and so presumably CAPP, can dephosphorylate the glycogen associated form of PP-1 ($PP\text{-}1_G$)(Dent *et al.*, 1990), thus interfering with insulin stimulation of $PP\text{-}1_G$. PP-2A also has the ability to dephosphorylate GSK3 (Murai *et al.*, 1996), thereby reversing the inactivation of GSK3 by insulin. Both of these are potential mechanisms which

would explain the inhibitory actions of ceramide on the observed changes in glycogen upon insulin administration, and could at least in part explain the inhibitory effect of TNF on the action of insulin.

A major limitation of the studies involving the actions of TNF on insulin-stimulated glycogen synthesis is that only one concentration of TNF was utilised. Although complete blockade of insulin-stimulated end point glycogen levels was not seen with TNF, it would be naive to assume that the response seen with this one concentration of TNF was submaximal or even maximal. Indeed, it is entirely possible that far lower concentrations of TNF could cause similar disruptions in insulin action. If the concentration of TNF used was grossly higher than that required to cause a maximal response, then one would expect that it would be harder to block this response than if a sub-maximal concentration of TNF were utilised. Therefore, any inhibitory actions of okadaic acid on the attenuation of the insulin response by TNF, could be masked by a high concentrations of TNF. Further investigations should continue focusing on the concentration dependency of this response with respect to TNF.

The results of this study are in contradiction to the observations of Kanety *et al.* (1995), who demonstrated that inclusion of okadaic acid, or inclusion of another phosphatase inhibitor, calyculin A, could increase the phosphorylation state of IRS-1 and convert it into an inhibitor of the insulin receptor. Therefore, in this system okadaic acid mimicked the observed insulin inhibitory actions of TNF. In my model okadaic acid did not inhibit insulin action, instead it blocked the inhibition of insulin-stimulated end point glycogen levels caused by ceramide. The reason for this apparent discrepancy is unclear, however, Kanety *et al.* (1995) used a 100-fold greater

concentration of okadaic acid than that used in my studies. At this concentration, okadaic acid is not selective for the PP-2A family of phosphatases, and may also inhibit PP-1 (Cohen *et al.*, 1989). Non-selective inhibition of phosphatases could explain the obvious differences between my study and that of Kanety *et al.* (1995).

End point glycogen levels after low glucose perfusion, in the absence of insulin were not different when comparing control and TNF treated groups, however these end point glycogen levels were already very low. Therefore, it is impossible to tell from this whether TNF could affect glycogen homeostasis in the absence of insulin. For this reason a separate set of experiments were conducted to see if TNF could affect glycogen breakdown. Hearts were perfused with substrate free Kreb's buffer and then freeze clamped at various time intervals and assayed for glycogen. One would expect no glycogen synthesis during these conditions due to lack of substrate, and glycogen breakdown would predominate. Glycogen depletion in control hearts under these conditions compared well with literature from other groups following similar protocols (Opie *et al.*, 1962; Sultan & Kahn, 1997). The fact that TNF did not increase the rate of glycogen breakdown, suggests that the effects of TNF on insulin-stimulated end point glycogen levels are an actual inhibition of the insulin-mediated response, as opposed to a non-specific action of TNF on glycogen degradation. Unfortunately, glycogen synthesis in the absence of insulin could not be achieved (data not shown), therefore a non-specific action of TNF on glycogen synthesis cannot be ruled out.

It has been suggested that in the absence of insulin, TNF can actually cause an increase in basal glucose uptake in various tissues (Meszaros *et al.*, 1987; Lang *et al.*, 1992). Indeed, in the investigation by Lang *et al.* it was shown that TNF could stimulate

peripheral glucose uptake, an effect which was seen in various tissues, including the heart. The results presented herein are in contrast to the studies mentioned above, as in the heart, under the conditions used, TNF did not stimulate basal glucose uptake. The reason why TNF did not increase basal glucose uptake in my investigations probably involves the time course used in this study when compared to the other investigations. During my experiments, TNF was in contact with the heart for a total of 80 min as opposed to 3 hr (Meszaros *et al.*, 1987) or 24 hr (Lang *et al.*, 1992).

A recent observation by Guo & Donner (1996) showed that brief treatment of 3T3-L1 adipocytes with TNF (15 min) could actually increase insulin-induced tyrosine phosphorylation of IRS-1, although chronic TNF treatment still caused insulin resistance. Guo & Donner did not actually investigate the functional consequences of this, but one would expect that this acute action of TNF would cause a potentiation of the insulin signal. I found no evidence that TNF could acutely potentiate insulin-stimulated glucose uptake in the isolated perfused heart.

With respect to contractility, TNF did not cause a negative inotropic effect in the low glucose perfusion model. This result is in contrast to other results presented herein, where acute negative inotropic responses to TNF were observed. The reason for this apparent discrepancy is not clear at present, however, as shown in figure 3.5 developed tension in control hearts dropped significantly upon perfusion with Kreb's buffer containing low glucose, when compared with normal glucose perfusion. This faster decline in cardiac viability, probably due to substrate deprivation, could have masked the expected negative inotropic action of TNF. Insulin initially showed a tendency to improve the developed tension, although this only reached statistical

significance at one time point in hearts where insulin was added at the start of the experiment. When insulin was added 15 min after recirculation, developed tension was significantly improved at later time point, however, hearts from this insulin treated group already appeared to have slightly increased developed tension. The improvement in developed tension seen with insulin was probably due to increased substrate availability for the heart, evidenced by the increase in glucose uptake observed with insulin. Interestingly, addition of TNF in the presence of insulin did decrease contractility with respect to insulin alone, this was regardless of the time when insulin was added. Thus, the slight improvement in contractility seen with insulin may have allowed the negative inotropic action of TNF to become apparent.

The observed effects of ceramide on developed tension in this model were interesting. C_2-ceramide, 1 μM, did not adversely affect developed tension, and may have even improved contractility at later time points. C_2-ceramide at higher concentrations, of 5 μM and 10 μM, caused a concentration-dependant negative inotropic response. A review of the literature suggests that three possible mechanisms could mediate this ceramide induced negative inotropic response. Firstly, ceramide is a direct metabolite of the SMase pathway, and upon activation of this pathway ceramide concentrations rise (Begum & Ragolia, 1996). Ceramide can then be subsequently metabolised to sphingosine by ceramidase (Kolesnick, 1991), another important enzyme in the SMase pathway. If, in cardiac myocytes, there is a basal activity of ceramidase, then a portion of the exogenously added ceramide could be converted to sphingosine. As discussed in more detail in the introduction (section 1.5.1) and later in this discussion (section 4.5), sphingosine can reduce the phenomenon of Ca^{2+}-induced Ca^{2+} release (Dettbarn *et al.*, 1994) as well as reducing L-type Ca^{2+} current (McDonough *et al.*, 1994), and this

would compromise cardiac contractility (Oral *et al.*, 1997). In addition to this, ceramide itself may alter cardiac function directly, by inhibiting the L-type Ca^{2+} current (Schreur & Liu, 1997). A third potential mechanism which could mediate the ceramide-induced negative inotropic response stems from the ability of ceramide to cause apoptosis of cardiac myocytes (Bielawska *et al.*, 1997). However, the time course required for ceramide-induced apoptosis is not consistent with the almost immediate negative inotropic actions of ceramide observed here. Commonly, several hours are required for ceramide to cause apoptosis (Obeid *et al.*, 1993; Jarvis *et al.*, 1994).

The blockade of insulin-stimulated glycogen synthesis could have important consequences for the heart, especially under conditions of substrate deprivation, where endogenous energy stores within the heart become of crucial importance. Although controversy exists concerning beneficial and detrimental effects of high cardiac glycogen content prior to ischaemic episodes, under certain conditions glycogen can certainly protect (Goodwin *et al.*, 1994; Cross *et al.*, 1996). However, contrary to this, preischaemic glycogen depletion in hearts has been shown to be protective during ischaemia (Neely *et al.*, 1984; Kupriyanov *et al.*, 1988). The distinction between these protective and detrimental consequences of cardiac glycogen levels appears to be a function of the severity of the ischaemic episode, whereby high glycogen levels are protective during relatively short ischaemic challenges and detrimental during prolonged ischaemia (Cross *et al.*, 1996). The most important consequence of TNF blockade of insulin-stimulated glycogen synthesis, however, could concern the role for glycogen as a reserve of energy to support sudden increases in heart work. After such increases in heart work TNF could compromise the recovery of cardiac glycogen

levels, and therefore subsequent increases in heart work may not be supported by cardiac glycogen as an energy source.

Inhibition of glycogen synthesis, by TNF, in the absence of an inhibition in glucose uptake, could have implications which are independent of those discussed above. Insulin stimulation of glucose uptake by the heart would lead to increased glycolysis, due to increased substrate delivery. Another action of insulin is to increase the activity of pyruvate dehydrogenase (Hughes *et al.*, 1980), the enzyme which is responsible for the conversion of the end product of the glycolytic pathway, pyruvate, into acetyl CoA. Acetyl CoA can then be oxidised via the citric acid cycle. However, if glycogen synthesis is blocked then proportionately more glucose would undergo glycolysis. This could saturate the pyruvate dehydrogenase enzyme and lead to increased metabolism of glucose to lactic acid, via lactate dehydrogenase metabolism of excess pyruvate levels. Increased lactate could then lead to acidosis within the heart.

4.4 TNF in the low glucose, low flow ischaemic heart

The above discussion concerns the effects of TNF on the perfused heart under substrate limiting conditions, and how the actions insulin were altered by TNF. To further these observations it was decided to investigate the actions of TNF under conditions of further substrate deprivation. Here a low flow, 2 $ml.min^{-1}$, model of perfusion was utilised, still under low glucose, 2 mM, conditions. The findings of these investigations can be summarised as follows: upon a change in flow rate from 10 $ml.min^{-1}$ to 2 $ml.min^{-1}$, there was a dramatic fall in parameters of developed tension and coronary perfusion pressure. Unexpectedly, TNF increased the contractility of hearts during this episode of low flow. Insulin also affected a slight protection from the decline in developed tension seen under low flow conditions, however this was only observed early after commencement of low flow. A marked contracture developed upon initiation of low flow. Insulin, in both the presence and absence of TNF, appeared to decrease the rate of formation of this contracture, but this effect was not proved to be significant. As in hearts perfused with low glucose insulin caused a significant increase in glucose uptake from the recirculating Kreb's buffer. Again insulin-stimulated glucose uptake was not altered by TNF. Initiation of low flow caused the release of lactate into the recirculating perfusate. Insulin, both in the presence and absence of TNF, significantly increased the accumulation of lactate in the Kreb's buffer. Release of lactate provides evidence that the low flow perfusion conditions were severe enough to constitute ischaemia, or at least hypoxia. Except for in control hearts, end point ATP and PCr levels were depressed after the low flow ischaemic episode in all groups, again indicating substrate deprivation. In control

hearts, ATP levels did not significantly reduce, although a definite trend could be seen. However, end point PCr levels actually appeared to increase. The ischaemic episode decreased end point glycogen levels when compared to preischaemic levels, however, in contrast to earlier observations, no differences were observed between treated groups and control groups.

During the ischaemic episode, the improvement in contractility seen with TNF was a surprise, and suggests that TNF can protect from the loss of function observed under these conditions. A review of the literature shows that protection by TNF from ischaemia or hypoxia is not a novel observation. In a study by Eddy *et al.* (1992) it was shown that pre-treatment of animals for 24 hr with TNF, resulted in protection from an *ex vivo* ischaemic insult to the heart. This injury was assessed by release of lactate dehydrogenase from the heart. These authors showed that 24 hr pre-treatment with TNF resulted in increased expression of manganous superoxide dismutase, thus it was speculated that protection from ischaemia afforded by TNF was due to increased resistance of the heart to oxygen free radicals. A second, more recent study has demonstrated that 12 hr pre-treatment of isolated cardiac myocytes with TNF resulted in protection from a continuous hypoxic challenge (12 hr) (Nakano *et al.*, 1998). In this study cell injury was assessed by lactate dehydrogenase release, $^{45}Ca^{2+}$ uptake and 3-[4,5-dimethylthiazol-s-yl]2,5-diphenyltetrazolium bromide (MTT) metabolism. It was concluded that an unknown mechanism was responsible for this protection, although TNF-induced expression of heat shock protein 72 may have played a role in the protection observed (Nakano *et al.*, 1998). It would be tempting to speculate that the apparent protection seen with TNF in my model of ischaemia is analogous to the protection in one or both of the studies described above. But I do not believe that this

is the case for the following reasons. In my studies, TNF was only present 25 min before the ischaemic insult, and was also present throughout the protocol. In both of the above studies several hours TNF pre-treatment was required for protection. Such long time periods as used in the studies discussed above would allow time for protein synthesis to increase the levels of manganous superoxide dismutase or heat shock proteins (Eddy *et al.*, 1992; Nakano *et al.*, 1998). Furthermore, it is unlikely that increased expression of manganous superoxide dismutase would even be protective in my model of low flow ischaemia. The reason for this is because hearts, even under these low flow conditions, were still being perfused, albeit at a lower than usual flow rate. This constant perfusion would be expected to wash any free radicals produced out of the system. I have no explanation for the observed increased contractility observed during low flow ischaemia with TNF. Insulin caused an early protection from the decline in developed tension seen upon initiation of ischaemia. This was probably due to the marked increase in glucose uptake, seen when insulin was present, which would have resulted in an increase in the substrate supply to the heart.

Almost at the onset of ischaemia a contracture developed, this was recorded as an increase in the baseline tension. The development of contracture was expected, and has been observed during other protocols of low flow ischaemia (Owen *et al.*, 1990). TNF did not alter this contracture, however, inclusion of insulin appeared to slow its development. Indeed, if insulin had protected the hearts from the observed contracture, then it would fit with data showing that rate of glucose uptake can regulate the onset of contracture (Owen *et al.*, 1990), where increasing glucose uptake in hearts subjected to global low flow ischaemia, protected from the development of contracture.

The lactate released from the ischaemic heart shows that the conditions used were severe enough to constitute ischaemia, or at least hypoxia. This is because under aerobic conditions glucose is metabolised by glycolysis to pyruvate, decarboxylation of pyruvate by pyruvate dehydrogenase causes the formation of acetyl-CoA, which can then be oxidised in the citric acid cycle. Under anaerobic conditions, oxygen required for the citric acid cycle is simply not present, and pyruvate is reduced to lactate. Therefore, increased release of lactate indicates hypoxic conditions. Insulin both alone and in the presence of TNF caused a significant increase in the release of lactate from hearts in my studies. This is consistent with the increase in glucose uptake induced by insulin. Increased glucose uptake would increase the flux of glucose through glycolysis and, hence, increased pyruvate production, thus more pyruvate would be converted to lactate.

As expected, end point glycogen levels in hearts, regardless of treatment, were markedly depressed after ischaemia, when compared with preischaemic levels, and this reflects the utilisation of this endogenous energy store within the heart. Considering the clear stimulatory effects of insulin on end point glycogen levels under low glucose conditions (section 4.4), it was rather surprising that inclusion of insulin into the Krebs buffer did not alter end point glycogen levels with respect to control hearts under low glucose, low flow conditions. A clue to a possible explanation of this observation is evidenced by increased lactate release from insulin treated hearts. This indicates that a large proportion of glucose taken up by the heart under these conditions is utilised, via glycolysis, for energy production. Under such conditions low rates of glycogen synthesis would be expected, due to lower concentrations of the substrates (G-6-P

and G-1-P) required for glycogen synthesis, despite the actions of insulin on the glycogen regulatory enzymes previously described. This theory makes sense when considering the survival benefits of increased energy production, versus that of increased energy storage, within substrate deprived hearts.

The changes in ATP and PCr were almost as expected. Ischaemia caused a decline in both ATP and PCr levels for all treatment groups, with the exception of ATP and PCr levels in control hearts. The decline in ATP and PCr is consistent with substrate deprivation was as expected (Van Binsbergen *et al.*, 1996). However, in control hearts end point PCr levels were not different from preischaemic values. This is curious as ATP levels in control hearts did appear to decline during the ischaemic episode, and one would expect that this would have been preceeded by a decline in PCr, hence, if ATP levels were lower than preischaemic controls then one would expect that PCr levels should also have been lower than preischaemic values. I have no explanation for this at this point in time, however, the number of hearts used to determine pre-ischaemic ATP and PCr levels was low, only four in each group. This may have lead to an artificially low estimation of preischaemic PCr levels from control hearts.

The increase in contractility observed with TNF suggests protection from the ischaemic insult, however, this increase in contractility was not accompanied by increased substrate supply to the heart, evidenced by the fact that TNF did not alter basal glucose uptake. If TNF, for some unknown reason, were to force the heart to do more work under conditions of limited substrate supply, as is suggested by the increase in developed tension seen, then it may be expected to have a deleterious action over a prolonged period of time, or during a reperfusion phase. Thus the actions of TNF

under these conditions should not necessarily be viewed as protective. Further studies should continue with the inclusion of a reperfusion phase after an ischaemic insult to see if the presence of TNF under these conditions would be beneficial or harmful. Studies from other groups suggest that the release of TNF is directly detrimental to a tissue subjected to an ischaemic episode, and protection from ischaemia can be seen by blockade of TNF actions or inhibition of TNF release (Colletti *et al.*, 1990; Squadrito *et al.*, 1993; Meldrum *et al.*, 1998).

4.5 TNF and contractile function of isolated perfused hearts under constant flow conditions

The aim of this study was to examine the mechanisms underlying the early cardiac depressant actions of TNF in the isolated perfused rat heart. The findings of the study presented herein can be summarised as follows: isolated hearts perfused under constant flow conditions with TNF showed a depression in left ventricular contractility, as indicated by the decrease in LVDP which was evident within minutes of TNF treatment and was sustained for the remainder of the experiment. This negative inotropic action was also evidenced by a depressed rate of both systolic contraction and diastolic relaxation in TNF treated hearts. TNF also caused a depression in the Starling curves, although this depression only became significant during Starling curves performed 135 min and 165 min after initial perfusion of the hearts, or 110 min and 140 min after addition of TNF. End point levels of glycogen, ATP, PCr, G-6-P, G-1-P, F-6-P and F-1,6-P were not altered in normal hearts after TNF addition. The observed depression in cardiac function was not potentiated by perfusion with Krebs buffer containing a high concentration of glucose, 25 mM. In hearts perfused with high glucose, TNF depressed end point ATP levels, but not glycogen, PCr, G-6-P, G-1-P, F-6-P or F-1,6-P levels.

The direct depression in LVDP seen with TNF in hearts perfused with Krebs buffer containing normal glucose, was not blocked by nitro-L-arginine, bosentan or indomethacin, which inhibit nitric oxide release, endothelin actions and cyclooxygenase mediated arachidonic acid metabolism respectively (Gross *et al.*, 1990, Clozel *et al.*,

1994, Vane, 1971). Thus NO, endothelins and arachidonic acid metabolites are unlikely to be involved in the TNF-induced decrease in LVDP. The negative inotropic actions of TNF were completely blocked by the ceramidase inhibitor NOE. This suggests that these actions of TNF are, at least in part, dependant on ceramidase converting ceramide to sphingosine, and is in agreement with the recent data of Oral *et al.*, 1997 and Bozkurt *et al.*, 1998. The fact that NOE also attenuated the negative inotropic actions of SMase provides indirect evidence that activation of the sphingomyelinase pathway can depress cardiac contractility in the whole heart, and that this effect can be blocked with an inhibitor of the enzyme responsible for the conversion of ceramide to sphingosine.

In the low glucose perfused heart TNF did not depress cardiac function, however, in hearts perfused with normal glucose TNF had a marked negative inotropic effect. Therefore the reason for investigating the effects of TNF in the isolated heart perfused with high glucose, 25 mM, containing Krebs buffer, was to investigate whether the TNF-induced negative inotropic effect was dependant on glucose. Perfusion of control hearts with high glucose did not affect the basal parameters of LVDP, and also did not alter the cardiac depression seen TNF. This adds further weight to the theory presented in section 4.3, where it was suggested that the faster decline in cardiac function seen in the low glucose heart could have masked the expected negative inotropic action of TNF. And suggests that the negative inotropic effects of TNF are independent of glucose.

As mentioned in section 3.6 and as will be mentioned later (section 4.6), administration of TNF to isolated hearts perfused under constant flow conditions caused an early and

sustained increase in coronary tone. By definition constant flow conditions ensure that coronary flow cannot change with coronary tone, and therefore one would not expect contractility to be altered by increased CPP caused by increased coronary tone. However, it is possible for the coronary circulation to alter cardiac function under constant flow condition via the "garden hose" effect (Katz, 1992b). With the "garden hose" effect increased CPP causes the coronary arteries to stretch the cardiac muscle. This in turn causes increased crossbridge cycling within cardiac myocytes, and, hence, can change cardiac contractility due to Starling's laws of the heart. Thus, it is important that the direct cardiac actions of TNF can be distinguished from the coronary actions. Several lines of evidence show that the depression in cardiac function observed with TNF is a direct action, and independent of the TNF-induced coronary constriction. Firstly, the stretching of cardiac muscle seen during increased coronary perfusion pressures would be expected to increase the force of contraction due to increased crossbridge cycling, however a depression in LVDP was observed with TNF. Secondly, indomethacin did not attenuate the negative inotropic actions of TNF, but, as discussed in section 4.6, did block the coronary constrictor actions of TNF. Thirdly, the experiments conducted with the stable thromboxane mimetic, U46619 (section 3.6.2), show that a similar coronary constriction to that seen with TNF, did not adversely affect contractile function under the constant flow conditions used in these experiments. Fourthly, administration of nitro-L-arginine also caused a vasoconstriction under constant flow conditions, however, this was of greater magnitude than that seen with either TNF or U46619, but again this was not accompanied by cardiac depression. These observations suggest a dissociation between the cardiac depression and the coronary constriction seen with TNF in this situation.

The ceramidase inhibitor, NOE, which attenuated the depressant actions of TNF under constant flow conditions, disrupts signalling through the SMase pathway by inhibiting the conversion of ceramide to sphingosine (Sugita *et al.*, 1975), an action which appears to be specific for the SMase pathway (Coroneos *et al.*, 1995). This suggests that the depressant effects of TNF are due to increased sphingosine production. Sphingosine has been shown to have the potential to disrupt cardiac myocyte Ca^{2+} handling, both by decreasing Ca^{2+} release from the sarcoplasmic reticulum (Sabbadini *et al.*, 1992, Dettbarn *et al.*, 1994, Webster *et al.*, 1994), and also by a depressing the L-type Ca^{2+} currents (McDonough *et al.*, 1994). I have shown that the addition of SMase also caused a decrease in cardiac function. However, it should be noted that the site of action of this exogenous SMase was not determined. The sphingomyelinase enzyme used in this study has a molecular size of 25 kDa (Matsuyama *et al.*, 1992), so it is likely that it will have access to the cardiac myocytes from the coronary circulation. Cardiac depression seen with SMase was also attenuated by NOE, suggesting activation of the SMase pathway, and release of sphingosine is responsible for its cardiac depressant action. However, it should be noted that the source of the SMase used in this study was bacterial in origin, and, hence, it is possible that it contained trace amounts of endotoxin. Contamination with endotoxin, and hence subsequent activation of constitutive NOS by endotoxin, could explain why NOE did not completely block the SMase-induced depression in cardiac function.

Whilst activation of the sphingomyelinase pathway by TNF has been known for some time (Dressler *et al.*, 1992, Schutze *et al.*, 1992), it has only recently been shown in cardiac tissue (Oral *et al.*, 1997). These authors demonstrated that TNF-induced

sphingosine accumulation was responsible for an early negative inotropic action of TNF in isolated feline cardiac myocytes. These authors also showed that the depressed cardiac function observed upon TNF administration, or addition of sphingosine, was accompanied by a decrease in the intracellular Ca^{2+} transient seen during systole. This is consistent with previous work from the same laboratory showing that depressed Ca^{2+} transients are responsible for the early negative inotropic action of TNF (Yokoyama *et al,*. 1993), and is also consistent with the known effects of sphingosine on Ca^{2+}-induced Ca^{2+} release (Webster *et al.*, 1994). My data strongly supports this work and indicates that similar mechanisms for TNF-induced early cardiac depression, as exist in the feline myocardium, also exist in the rat heart.

The early depression in function was not dependent on NO because it was not altered by the NOS inhibitor nitro-L-arginine. This is in contrast to the observations of Finkel *et al.* (1992), but is in agreement with other reports showing that the early direct decrease in cardiac function produced by TNF was not prevented by NOS inhibition (Yokoyama *et al.*, 1993; Oral *et al.*, 1997, Bozkurt *et al.*, 1998). Schulz *et al.* (1995) showed that TNF and IL-1β-induced synthesis of iNOS was responsible for a late depression (> 2 hr) in function in the isolated perfused rat heart. To my knowledge, alterations in cardiac function involving iNOS take place over a long period of time due to the requirement of protein synthesis involved for iNOS expression (Schulz *et al.*, 1992; Shindo *et al.*, 1994; Balligand *et al.*, 1994; Pinsky *et al.*, 1995), this clearly is not the case in my system where a negative inotropic action was observed within 20 min. In contrast to our experiments, Schulz *et al.* (1995) did not observe an early depression in cardiac contractility, in fact a small increase in cardiac work was seen. This is a discrepancy for which I have no explanation.

My results show that the release of endothelin, which occurs after treatment of animals with lipopolysaccharide or TNF (Hohlfeld *et al.*, 1995, Klemm *et al.*, 1995a,b), does not play a role in the early negative inotropic action of TNF under constant flow conditions. This is evidenced by the fact that the $ET_{A/B}$ receptor antagonist, bosentan, did not alter the TNF-induced decrease in LVDP.

When the recirculating perfusate from one heart was passed through another, there was a decrease in contractility on the second heart. This adds further evidence to the idea the process of recirculation caused a faster decline in contractility due to the build up of metabolites in the recirculating perfusate (section 4.1). Also shown during these experiments was that the recirculating perfusate from a TNF treated heart did not cause a greater decline in contractility than the recirculating perfusate from a control heart. This shows that the depression in function seen with TNF was not the result of a build up of a substance released by TNF in the perfusate, but was probably the result of a direct action of TNF.

The results obtained during perfusion if recombinant rat TNF were very similar to those obtained with recombinant human TNF, an early depression in cardiac function seen within minutes of TNF perfusion. Thus I feel justified in using recombinant human TNF in the rat isolated perfused heart

In summary, I add further evidence in confirmation of the studies by Oral *et al.* (1997) and Bozkurt *et al.* (1998) showing that a direct and early negative inotropic action of TNF in the isolated perfused rat heart is likely to be mediated via activation of SMase.

4.6 Actions of TNF on the coronary circulation in hearts perfused under constant flow conditions

The aim of this study was to observe and characterise the acute actions of TNF on the coronary circulation of the isolated perfused rat heart under recirculating conditions. The major findings of these experiments can be summarised as follows: TNF caused an initial rise in CPP which was sustained throughout the experiment. This increase in coronary tone appeared to be mediated by the vasoconstrictor prostanoid, thromboxane A_2, because it was completely abrogated by two different thromboxane antagonists, as well as by the cyclooxygenase inhibitor indomethacin. The inhibition of TNF-induced coronary constriction by indomethacin is especially interesting considering the lack of effect of indomethacin on the direct depression of left ventricular contractility. As with the TNF-induced depression in cardiac function, the increase in coronary tone observed with TNF was inhibited by the ceramidase inhibitor NOE, suggesting that sphingosine is also involved in the coronary constriction induced by TNF. The $ET_{A/B}$ receptor antagonist bosentan did not inhibit this action of TNF, indicating that the potent vasoconstrictor peptide, endothelin, is not involved in the immediate actions of TNF in the coronary circulation of the rat isolated perfused heart.

Other studies have shown that TNF can activate PLA_2 (Clark *et al.*, 1988; Hayakawa *et al.*, 1993) and the SMase pathway (Kolesnick, 1991). However, I am unaware of any studies examining the effects of TNF on these pathways in the isolated perfused rat heart. Our studies show a role for these pathways in the responses of a whole organ to TNF. The fact that the ceramidase inhibitor NOE (Sugita *et al.*, 1975) and two

thromboxane antagonists, GR32191 and ZD1542, inhibited the coronary constrictor actions of TNF raises a number of possibilities. Three potential explanations could account for the observations that have been made: firstly, TNF activates SMase to release sphingosine, which in turn causes the release of thromboxane A_2, to induce coronary vasoconstriction; secondly, TNF could activate the PLA_2 pathway, resulting in thromboxane A_2 synthesis, this may then release sphingosine to cause vasoconstriction; a third possibility is that two entirely different pathways are activated by TNF, which act in synergy to cause the constriction. It is not possible to tell from these studies which is the correct suggestion, although the third possibility is unlikely because the thromboxane antagonists and NOE both produced the same physiological response, complete inhibition of the TNF action, indicating that a sequential activation of the two pathways is more likely. When these data are compared with the data describing the actions of TNF on contractility of the isolated perfused heart, then indomethacin blocked the coronary constriction, but did not alter the TNF-induced depression in cardiac function. This is interesting as it suggests that in the myocardium, cyclooxygenase mediated breakdown of AA is not responsible for the activation of the SMase pathway. If this is also the case with respect to the coronary constrictory actions of TNF, then it indicates that sphingosine release is proximal to the release of thromboxane A_2. In addition, Murohara *et al.* (1996) showed that exogenous addition sphingosine caused a coronary constriction in isolated porcine coronary arteries, which was completely inhibited by indomethacin, suggesting breakdown of AA, and subsequent production of a vasoconstrictory prostanoid. This taken together with the well known and characterised vasoconstrictory actions of thromboxane A_2 (Moncada & Vane, 1978) imply that thromboxane A_2 is the final mediator of the TNF-induced coronary constriction seen in my system.

Germane to this discussion, exogenous sphingosine has been shown to potentiate cytokine-induced prostaglandin production by increased activation of both PLA_2 and cyclooxygenase enzymes in fibroblasts (Candela *et al.*, 1991; Ballou *et al.*, 1992). In the study by Candela *et al.* (1991), TNF stimulated the production of prostaglandin E_2 by human fibroblasts. Relatively low concentrations of sphingosine, 0.5 μM, caused remarkable potentiation of the TNF response, and higher concentrations, 10 μM, increased prostaglandin E_2 production by 18-fold. (Candela *et al.*, 1991). This was shown to be accompanied by synergistically increased activity of PLA_2. Ballou *et al.* (1992) showed very rapid induction of cyclooxygenase protein synthesis (1-2 hrs) after sphingosine treatment. However, it is unlikely that induction of cyclooxygenase protein synthesis is involved in the actions of TNF observed herein due to the time required for protein synthesis. The vasoconstrictory action of TNF on the coronary circulation in the isolated perfused heart was observed within 5 min of TNF administration.

Interestingly, arachidonic acid has been shown to cause the stimulation of SMase and, hence, breakdown of sphingomyelin (Jayadev *et al.*, 1994), and these authors postulated that TNF-induced activation of PLA_2 was essential for TNF instigated activation of SMase. Taken together with the observations of Candela *et al.* (1991), these results lead to the intriguing possibility that a self potentiating mechanism exists in TNF signal transduction, whereby release of arachidonic acid, by TNF activation of PLA_2, could cause release of sphingosine, via activation of SMase, which could, in turn, increase arachidonic acid breakdown as well as causing further activation of PLA_2.

Several recent reports from the same group have shown that following removal of hearts, 15 to 30 minutes, after administration of lipopolysaccharide or TNF to the whole animal, there is a marked increase in the coronary tone in the isolated rat heart (Hohlfeld *et al.*, 1995; Klemm *et al.*, 1995a, Klemm *et al.*, 1995b). This coronary vasoconstriction was associated with increased circulating endothelin levels and was attenuated by the selective ET_A receptor antagonist, FR139317 (Hohlfeld *et al.*, 1995). The experiments described herein serve to extend the observations made by the above groups, as the ET_A receptor antagonist used did not completely abolish the coronary constriction observed (Hohlfeld *et al.*, 1995). Thus, a component of the coronary constriction observed may have been due to TNF-induced activation of the SMase pathway, and subsequent release of thromboxane A_2. The fact that in my experiments the non-specific ET receptor antagonist, bosentan, at a concentration that attenuates the coronary constrictor response to 100 pM endothelin-1, did not affect the TNF-induced rise in CPP, suggests that the release of endothelins are not involved early action of TNF seen in my studies. The reason for this apparent conflict is not at present clear, however, there are obvious differences between protocols followed. In the reports by the Vane group, the source of the proposed endothelins was not identified, and it is possible that overproduction of endothelins by organs other than the heart could have caused the large coronary vasoconstriction observed, clearly this would not apply to my studies, where TNF was added directly to the isolated heart perfused *in vitro*.

Metabolites of arachidonic acid have been proposed to play a role in the cardiovascular alterations observed during septic shock. Indeed, it has been postulated that the vasoconstrictor actions of released thromboxane A_2 may in part compensate for the

profound peripheral vasodilation seen in shocked states (Cirino *et al.*, 1996). But conversely, inhibitors of arachidonic acid metabolism and thromboxane synthesis have been shown to be protective during shock (Fletcher & Ramwell 1977; Butler *et al.*, 1983; Bult *et al.*, 1985; Boughton-Smith *et al.*, 1989; Mozes *et* al., 1991). Subsequent experiments discussed in this report (section 4.7) demonstrate that, under certain conditions, these changes in coronary tone can act to alter cardiac function. Therefore, thromboxane A_2-induced coronary vasoconstriction would at least in part explain the observed protection of inhibitors of thromboxane A_2 action seen during shock.

A potential limitation of the work presented herein arises from a question over the specificity of the ceramidase inhibitor NOE, as it has been reported to inhibit cell swelling as well as Ca^{2+} release from isolated mitochondria, leading to inhibition of Ca^{2+}-dependant activation of PLA_2 (Epps *et al.*, 1982, Broekemeier *et al.*, 1985). Inhibition of Ca^{2+}-dependant activation of PLA_2 could account for the inhibitory actions of NOE on thromboxane A_2 release, however, the concentrations of NOE required for the above effects were far higher (200 μM) than those used in my experiments (1 μM), and to my knowledge the concentration used in my experiments does not inhibit PLA_2. NOE has also been shown to specifically inhibit the activity of growth factors such as platelet-derived growth factor which utilise the sphingomyelinase pathway but not for those which employ other pathways, e.g. ET-1 (Coroneos *et al.*, 1995), therefore, I feel justified in using this synthetic ceramide analogue as a specific inhibitor of ceramidase at the concentration used.

Due to the structure of the coronary circulation within the whole heart, where coronary arteries penetrate deep into the ventricular walls, coronary resistance can be

highly influenced by intramyocardial pressures (Katz, 1992c). Therefore, it is possible for changes in cardiac contractility to alter coronary tone, and so CPP. Except for GR32191, none of the inhibitors or antagonists used adversely effected cardiac contractility. GR32191 caused a marked bradycardia which was accompanied by a slight increase in cardiac contractility. In spite of this, GR32191 affected the same physiological antagonism, of the TNF-induced coronary vasoconstriction, as ZD1542. To this end I believe that the observations made herein are a result of the actions of the drugs used on the coronary circulation, and are independent of changes in cardiac contractility. The bradycardia seen with GR32191, was probably due to non specific actions of this drug, because the other thromboxane antagonist ZD1542 did not alter heart rate.

In the absence of TNF, and after recirculation, basal coronary tone slowly increased with time. This slow rise in CPP was significantly attenuated by both of the thromboxane A_2 inhibitors, GR32191 and ZD1542, suggesting that in the isolated perfused rat heart there is a constant, endogenous release of thromboxane A_2. Pomposiello *et al.*, (1997) have recently provided evidence to support this suggestion. However, in apparent contrast to this, indomethacin, which would be expected to inhibit thromboxane production, did not alter CPP in the absence of TNF. This could be explained if there is a basal turnover of both vasodilatory prostaglandins and constrictor thromboxane A_2, inhibiting the production of both of these might have no net effect of CPP. Blockade of thromboxane receptors and thromboxane synthesis could lead to an imbalance in the opposing forces and, hence, coronary dilation due to the unopposed release of vasodilatory prostaglandins.

In summary, TNFα caused an acute increase in coronary tone, evidence is presented to implicate sphingosine and thromboxane A_2 in this response. If this were to occur *in vivo*, it would lead to a decrease in blood supply to the heart, and so could contribute to the serious cardiovascular consequences of septic shock, where cardiac output is an essential factor in vital organ perfusion.

4.7 Actions of TNF in isolated hearts perfused under a constant head of pressure

As discussed above, I have shown that in the rat isolated perfused heart, under conditions of constant flow, addition of TNF can result in an early and direct depression in cardiac function as well as an increase in CPP, indicative of coronary vasoconstriction. It was decided to perfuse hearts with a constant head of pressure in order to investigate whether the increase in coronary tone, observed under constant flow conditions, would also be observed under constant pressure perfusion. When hearts are perfused under a constant head of pressure, then coronary flow rate is allowed to vary with the tone of the coronary vessels. Therefore a coronary dilation results in an increase in coronary flow, and a coronary vasoconstriction results in a decrease in flow. A decrease in coronary flow would be expected to indirectly alter contractile function through decreased substrate supply to the heart, i.e. glucose and O_2. Evidence that decreased flow rate can adversely affect contractile function was presented in section 3.4, where it was shown that during a low flow ischaemic episode, under constant flow conditions, a marked decline in the force of contraction was observed. Therefore the ultimate aim of the studies utilising a constant pressure perfusion model was to see if the coronary constriction observed with TNF could impair contractile function, and hence synergise with the direct negative inotropic actions of TNF resulting in a more severe depression in cardiac contractility.

The results of this study can be summarised as follows: as expected, upon administration of TNF to hearts perfused under a constant head of pressure there was a

decrease in the coronary flow rate, indicative of constriction of the coronary vessels. This coronary constriction was observed within 5 min, and appeared to be maximal after about 10 min. This time course was consistent with the increase in CPP observed in hearts perfused, with TNF, under constant flow conditions. TNF caused a depression in left ventricular contractility, which was evident within 10 min of TNF administration. The decline in cardiac function observed here paralleled the decrease in coronary flow. This differed from the depression in function observed under constant flow conditions where significant depression in left ventricular contractility was only seen after 20 min perfusion with TNF. The depression in contractility under constant pressure conditions was also more severe than seen with TNF under constant flow conditions. As with previous studies, NOE blocked both the decrease in coronary flow as well as the depression in cardiac function, again implicating sphingosine in these actions of TNF. Starling curves performed in the presence of TNF were markedly depressed when compared to control hearts. This depression in the Starling response was also inhibited by prior addition of NOE.

Thus, it was shown that the actions of TNF on the coronary vasculature could indirectly depress left ventricular contractility. The contractile dysfunction seen with TNF under these conditions was quicker in onset and of greater severity to the direct negative inotropic effects of TNF under constant flow conditions. This greater depression in cardiac function appeared to be a consequence of the coronary constriction. Other work from this laboratory has shown that, under constant flow conditions, the vasoconstrictory peptide ET-1 could depress cardiac contractility as a consequence of the coronary constrictory actions (Lal, *personnal communication*). This is in spite of the direct positive inotropic actions of ET-1 (Kramer *et al.*, 1991).

The implications for the results obtained here are important. If this depression in cardiac function were to occur during septic shock, then it would undoubtedly contribute to the severe hypotension observed in shocked states (Tracey *et al.*, 1987). Thus if these actions of TNF could be blocked, then increased cardiac function would be expected to increase blood pressure and hence improve vital organ perfusion. However, clinical application of this work with respect to septic shock, may not be feasible. This is because TNF is one of the earliest mediators released during shock. During experimental shock induced by infusion of live bacteria, TNF appears in the blood stream after about 45 min, peaks after 90 min, and returns to baseline after 6 hr (Redl *et al.*, 1991; Fischer *et al.*, 1992). The rapid pharmacokinetics of released TNF makes the window of opportunity for treatment very small and it is unlikely that diagnosis would occur in time for blockade of TNF actions to be useful.

As mentioned earlier, raised serum levels of TNF can be seen in cardiac diseased states such as chronic heart failure (Levine *et al.*, 1990; Ferrari *et al.*, 1995) and ischaemic heart disease (Vaddi *et al.*, 1994). Serum levels of TNF observed in these studies are unlikely to be high enough to cause the direct negative inotropic action of TNF observed in my studies, but the production of TNF by both cardiac myocytes (Kapadia *et al*,. 1995; Benigini *et al.*, 1996; Meldrum *et al*,. 1998; Wagner *et al.*, 1998) and resident cardiac mast cells (Frangogiannis *et al.*, 1998), may cause local TNF levels within cardiac tissues to rise to disproportionately higher levels than observed in the sera of patients with cardiac disorders. Under these conditions, the direct negative inotropic actions of TNF, via the activation of the SMase pathway, could be physiologically relevant. Also as mentioned in the introduction, naturally occurring

buffers to TNF do exist and high concentrations of "shed" TNF receptors can act to neutralise circulating levels of TNF (Tracey & Cerami., 1993). This statement is of particular interest when considering the production of TNF by the heart. By virtue of the size of the circulating "shed" TNF receptors (55 kDa and 75 kDa) they may not penetrate the interstices of some tissues and thus would not be expected to cross the coronary circulation (Kapadia *et al.*, 1995). Therefore, TNF produced by the heart would not be susceptible to the potential buffering actions of "shed" TNF receptors. If this were the case then it would only apply to the direct cardiac depressant actions of TNF and not the coronary constrictor actions, because to cause a coronary constriction TNF would have to be present within the coronary circulation, and would thus be susceptible to buffering by these shed receptors.

The protection from the TNF-induced depression in cardiac function affected by NOE is interesting for two reasons. Firstly, as discussed above, the observed actions of NOE implicates activation of the SMase pathway, and subsequent sphingosine production, in the negative inotropic actions of TNF. The second reason stems from a series of early studies concerning NOE itself. It has shown that N-acylethanolamines, of which NOE is one, can accumulate in the infarcted canine myocardium (Epps *et al.*, 1979; Epps *et al.*, 1980). Subsequent studies from the same group investigating a potential role for these accumulated N-acylethanolamines showed that NOE could protect against a hypoxic challenge to both guinea-pig atrial and ventricular strip preparations (Epps *et al.*, 1983). Thus, it was proposed that N-acylethanolamines produced by the ischaemic or infarcted heart, could act as endogenously produced protective agents. These observations are interesting when taken in the context of both the work presented herein, and publications showing the release of TNF during cardiac ischaemia

(Squadrito *et al.*, 1993; Meldrum *et al.*, 1998). In the first of these studies it was shown in an anaesthetised rat model of coronary artery ligation that serum TNF levels were markedly increased upon release of the coronary artery ligature. Immunisation with a hyperimmune serum containing antibodies against TNF showed a significantly increased survival rate for experimental animals (Squadrito *et al.*, 1993). Also Meldrum *et al.* (1998) showed that pre-treatment with adenosine protected the ischaemic hearts, an effect which was accompanied by reduced TNF release. Thus it appears that endogenous inhibitors (N-aceylethanolamines) of TNF-mediated sphingosine production, are produced during ischaemia or infarction, and could act to attenuate the potentially deleterious actions of TNF released under such conditions.

There has been much controversy concerning the cardiac actions of TNF, and indeed many authors have not been able to detect an early negative inotropic effect. Of these, the paper by Schulz *et al.* (1995), is probably the most important when considering the experiments presented herein. The investigations by Schulz *et al.* (1995), used an rat isolated working heart, under recirculating conditions, to examine the effects of TNF and IL-1β. No early negative inotropic effect of these cytokines was observed, even though the concentration of TNF used was exactly the same as in my studies, 20 $ng.ml^{-1}$. Thus my results are in contrast to those reported by Schultz *et al.* As discussed in section 4.5, this discrepancy is hard to resolve. However, one possible explanation could involve the concentrations of the phospholipid, sphingomyelin, present in the plasma membrane. Sphingomyelin is a dynamic phospholipid. In this regard, it has been shown that plasma membrane levels of sphingomyelin can change quite dramatically with both with the age (Yechiel *et al.*, 1985; Yechiel & Barenholz, 1986) and diet (Parrish *et al.*, 1997). Generally, sphingomyelin levels in the heart rise

with age, 18-month old rats have approximately 1.5-fold more membrane sphingomyelin that 3-month old rats (Yechiel & Barenholz, 1986). In cardiac myocytes, *in vitro*, this can also occur, whereby 15-day old cultures of neonatal cardiac myocytes contain more sphingomyelin than 5-day old cultures (Yechiel *et al.*, 1985). Similarly, a diet containing high cholesterol can lead to increased sphingomyelin levels in cells (Geelen *et al.*, 1995; Keelan *et al.*, 1997). Thus increased membrane sphingomyelin levels would lead to greater substrate for the SMase enzyme and presumably increased production of the second messengers of the SMase pathway, ceramide and sphingosine. Therefore different starting parameters with respect to membrane sphingomyelin levels, caused by either diet or age, could possibly explain the controversy surrounding the acute cardiac actions of TNF even in models which appear the same.

If this theory can be extrapolated into humans, then the consequences could be very important. Cardiac disease states are observed most often in the ageing population, where increased membrane sphingomyelin levels would be expected. Therefore, TNF released in cardiac disease states (Levine *et al.*, 1990; Arbustini *et al.*, 1991; Smith & Allen, 1992; Latini *et al.*, 1994; Vaddi *et al.*, 1994; Ferrari *et al.*, 1995) may have a greater than expected deleterious cardiac action, due to the increased membrane sphingomyelin levels.

4.8 Actions of sphingosine in the isolated perfused heart

In the studies described above it was shown that TNF could cause a direct depression in cardiac function, and a coronary constriction in the isolated perfused rat heart. The inhibition of these responses by the ceramidase inhibitor, NOE, implicated the ceramidase enzyme as an essential mediator of this response. Therefore, sphingosine was proposed as a mediator of the observed actions of TNF. In this regard, experiments were conducted to try to mimic the actions of TNF in the isolated perfused heart by exogenous addition of sphingosine. Concentration-dependent actions were observed, where sphingosine caused an increase in CPP, as well as a fall in LVDP. However, unlike TNF, application of sphingosine resulted in a dramatic increase in LVEDP.

Another discrepancy between the actions of sphingosine, and those of TNF, were in the observed coronary constriction seen. The coronary constriction with sphingosine followed slower kinetics than that with TNF. This was unexpected, as if sphingosine were the mediator of the TNF response, then one would expect that the response to sphingosine would be quicker in onset because there would be no need for TNF receptor binding, subsequent activation of SMase and breakdown of sphingomyelin to ceramide and then to sphingosine. A potential explanation for the differences in the coronary responses to TNF and sphingosine could lie with another discrepancy observed between TNF and sphingosine. Unlike TNF, the increase in CPP seen with sphingosine was not sensitive to inhibition by either indomethacin or ZD 1542, suggesting that cyclooxygenase metabolites of AA, especially thromboxane A_2, are not

involved in the constriction. As mentioned in the introduction and discussed in section 4.6, TNF causes the activation of PLA_2, thus releasing AA. It seems likely that prior activation of PLA_2 by TNF is required for the constrictory responses of sphingosine to become apparent. If this were the case, then the parallel activation of the PLA_2 and SMase pathways are likely to essential for the TNF mediated coronary constriction. It appears that the slower increase in CPP seen with sphingosine alone is due to an action of sphingosine which is independent of the PLA_2 pathway. The reason why there was not an indomethacin insensitive portion of the coronary constriction seen with TNF is probably because the concentration of sphingosine required to participate in the TNF response is lower than that required to cause a coronary constriction independent of TNF.

The fact that the coronary constriction seen with sphingosine was not blocked by indomethacin in my experiments is in contrast to the results of Murohara *et al.* (1996), who showed that in porcine coronary artery preparations, sphingosine caused a constriction, which could be inhibited by indomethacin. The reason for this difference is unclear, however a coronary artery ring preparation utilised by Murohara *et al.* is very different from the isolated perfused heart. In the isolated perfused heart, pressor responses observed are likely to be a result of changes in the deep resistance beds of the coronary circulation. A coronary artery ring preparation is derived from larger conductance vessels within the coronary circulation, as opposed to the deep resistance vessels. Thus, differential responses between different anatomical regions of the coronary circulation could explain the discrepancy between the results of Murohara *et al.* (1996) and those presented herein.

Application of sphingosine, ≥ 3 μM, to perfused hearts caused a large increase in LVEDP, or contracture. This made any measurements of LVDP hard to interpret due to the close relationship between LVEDP and LVDP, therefore although LVDP did decrease, it was uncertain if this was a consequence of this large increase in LVEDP. Thus, it is hard to ascertain whether sphingosine mimicked the negative inotropic actions of TNF. A very interesting observation concerning the contracture seen with sphingosine was that in hearts where the contractility was measured using both a hook and an intraventricular balloon, the contracture was not seen as an increase in baseline hook tension, only as an increase in LVEDP. This suggests that the contracture was not of the same type as observed in the low flow ischaemic heart, as in the low flow ischaemic heart, an increase in baseline tension was seen. Due to the disruptions in Ca^{2+} handling often seen with sphingosine (Sabbadini *et al.*, 1992; Dettbarn *et al.*, 1994; McDonough *et al.*, 1994; Webster *et al.*, 1994; Oral *et al.*, 1997), then it is plausible that exogenous sphingosine could cause this contracture by causing a Ca^{2+} overload, resulting in high diastolic intracellular Ca^{2+} and reduced relaxation of the myofillaments. But as mentioned before, sphingosine would be expected to decrease Ca^{2+} release from the sarcoplasmic reticulum, and not increase resting Ca^{2+} concentrations. Also, after perfusion with sphingosine, hearts did not have a so called "stone heart" appearance, which is a characteristic of a Ca^{2+} overload. Therefore it seems reasonable to assume that the developed contracture was not due to a massive elevation in resting Ca^{2+} levels. The mechanism responsible for the contracture seen with sphingosine deserves further investigation.

The developed contracture seen with sphingosine was not observed with TNF. The reason for this is also unclear, but suggest that either the final mediator of the TNF-

induced disruptions in ventricular contractility is not sphingosine, or that the contracture was the result of an non specific action of exogenous sphingosine. I believe that it is due to a non specific of sphingosine, maybe even on non muscle cells within the heart. It has been shown that TNF activates the SMase pathway in a cell free system (Dressler *et al.*, 1992), suggesting very tight coupling of the TNF receptors to the enzymes responsible for the SMase pathway. Thus one would expect the signal generated from the TNF receptors to be fairly localised, and so to target specific effectors of the SMase response, in this case the sarcoplasmic reticulum. However, application of exogenous sphingosine to the whole heart would be expected to swamp all parts of the heart, thus explaining any non specific effects. Further work should continue with the aim of clarifying the precise mechanisms behind the dramatic effects of sphingosine in isolated perfused hearts.

4.9 Summary

This project was designed to investigate the actions of TNF in the rat isolated perfused heart. During this study many interesting results have been achieved, some of which were expected, some which were not.

I have demonstrated that TNF did not alter the recovery of hearts after brief acidotic challenges.

I have shown that, under certain conditions TNF has the ability to differentially alter the actions of insulin in the isolated perfused heart. Namely, TNF attenuated insulin-stimulated glycogen synthesis, without disrupting insulin-stimulated glucose uptake. This was an action which was mimicked by ceramide. Okadaic acid blocked the actions of ceramide in insulin-stimulated glycogen synthesis, but only partially attenuated those actions of TNF.

TNF was shown to cause an increase in the contractility of heart perfused under low flow, low glucose conditions.

I have also shown that in the isolated perfused heart TNF caused both an early and direct negative inotropic action and I have presented evidence to suggest a role for sphingosine and activation of the SMase pathway in this action. In addition to this, TNF caused a very quick coronary constrictor response which, after pharmacological investigation, was shown to be probably due to concurrent activation and interaction

between the PLA_2 and SMase pathways. When perfused under conditions of constant pressure, this increase in coronary tone could add to and potentiate the direct cardiac depressant actions of TNF.

Exogenous addition of sphingosine caused profound alterations in cardiac function. A large increase in coronary tone was observed, which was insensitive to inhibition by indomethacin and ZD 1542. In addition to this, higher concentrations of sphingosine caused a severe cardiac contracture to develop, which was accompanied by a large depression in LVDP. However, due to the contracture, it was impossible to interpret this decline in cardiac function.

4.10 Future work

Further investigation into the actions of TNF on insulin sensitivity should be carried out. Different concentrations of TNF should be utilised to determine a precise concentration-response. If the concentration used in these studies were supermaximal then experiments with okadaic acid should be repeated.

Further studies concerning the actions of TNF during ischaemic conditions should be examined. A recovery phase should be included into the experiments utilising low flow conditions to see if the apparent protective actions of TNF in this model are truly protective.

Many questions were raised by the perfusion of whole hearts with sphingosine, and work is currently continuing in this laboratory, to investigate these actions. The current work involves investigations into the actions of sphingosine in isolated muscle preparations such as papillary muscles and atria in order to see if the actions of sphingosine in the whole heart can be reproduced in isolated muscles. If so then this would give a simpler model, than the whole heart, to study these interesting actions of sphingosine.

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6 Appendix, publications

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